

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No. : 09/973,088
Applicant : Marie B. CONNETT-PORCEDDU
Filed : 10 October 2001
TC/A.U. : 1638
Examiner : Stuart F. Baum

Confirmation No. 4800

Docket No. : 2411-110
Customer No. : 6449

COPY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER RULE 132 OF MARIE B. CONNETT-PORCEDDU

Dear Sir:

I, Marie B. Connett-Porceddu, declare as follows:

1. I am the inventor of the subject application.
2. My education and experience are as follows. I received a Bachelor of Arts degree in Biology from Humboldt State University in 1984 and a Doctorate degree in Botany from Cornell University in 1991. I have been employed by Arborgen, LLC, which is a joint venture including Westvaco Corporation, the assignee of the present application, from August, 2002 to present. I was employed as a Scientist and Senior Scientist with the role of the Mission Leader, Pine Tissue Culture and Transformation with Westvaco Corporation from 1998 to August, 2002. I was employed by Fletcher Challenge Forests from 1994 to 1997, first as a Molecular Biology Manager and then as a Biotechnology Manager. I was employed by New Zealand Forest Research Institute from 1992 to 1994 as Program Manager, Molecular Biology. I have been involved with tissue culture and transformation of pines since 1992.

3. I understand that the claims recite (a) a method for regenerating transgenic plants of pine of the genus *Pinus* subgenus *Pinus* (claims 1-9 and 11-38), (b) a method for minimizing damage to transformed cell of the pine of the genus *Pinus* subgenus *Pinus* following infection by

Agrobacterium (claims 39-43 and 45), (c) a method for pine cell tissue culture of pine cells of the genus *Pinus* subgenus *Pinus* (claims 46-51), (d) a method for selecting transformed cells of pine of the genus *Pinus* subgenus *Pinus* (claims 52-57), and (e) a method for eradicating *Agrobacterium* from cells of pine of the genus *Pinus* subgenus *Pinus* (claims 58-62). The method of claims 1-9 and 11-38 provides for enhanced transformation and regeneration of transformed embryogenic pine tissue in which the pine is of the genus *Pinus* subgenus *Pinus*. The remaining methods relate to various aspect of this method.

4. I have recently reviewed this application and the Office Action mailed January 28, 2003. I have also recently reviewed the Levee et al. (*Molecular Breeding* 5:429-440, 1999) reference cited in the Office Action. I have also reviewed amended claims to be filed with this Declaration.

5. I understand that the Examiner has stated that the claimed invention is unpatentable because it would have been obvious to persons skilled in the art to "use the method of Levee et al. and to optimize this method by optimization of process parameters that would not confer patentable distinction on the claimed invention." Office Action at page 4. I also understand that the Examiner has argued, with respect to Applicants' prior arguments of differences between hard and soft pines made to distinguish the invention from Levee et al., that "Applicants' mere unsupported assertions that hard pines are harder to transform than soft pines are not sufficient" Office Action at page 4. Finally, I understand that the Examiner has argued that "[n]owhere in the Levee et al reference do they mention the difference in regenerability of hard versus soft wooded pines." Office Action at page 4.

6. Applicants have invented a method which provides for enhanced transformation and regeneration of transformed embryogenic pine tissue in which the pine is of the genus *Pinus*, subgenus *Pinus*, as well as various aspects of this method. Pines of the subgenus *Pinus* are "hard pines". The method involves minimizing damage to cells subsequent to *Agrobacterium* infection and rapidly selecting transformed cells. The transformed cells are cultured to produce transgenic somatic embryos which are then germinated to produce transgenic plants. Applicants discovered that, through the use of the disclosed and claimed method and its various aspects, they were able to transform and regenerate transgenic pine plants of the *Pinus* subgenus, i.e. hard pines. Applicants'

invention allowed for the first time the transformation via *Agrobacterium* followed by regeneration of transgenic plants of pine of the subgenus *Pinus*, i.e., hard pines, especially at significant frequency.

7. Levee et al. discloses the transformation and regeneration of pine of the subgenus *Strobis* which, according to this reference, "is the first work on genetic transformation on **this pine species** as well as the first report of successful stable genetic transformation of **a pine species** using a disarmed strain of *A. tumefaciens*". (See page 36, first paragraph of Discussion, emphasis added). Levee et al. does not disclose the transformation and regeneration of pines of the subgenus *Pinus*. Nor would a skilled artisan expect that the method disclosed by Levee et al. for soft pines could be used or routinely modified for use with hard pines.

8. Specifically, it was well known at the time of the present invention that there were differences between soft pines and hard pines. These differences were seen in transformation and regeneration methods for soft pines and hard pines, such that there was no expectation of success with respect to the transformation and regeneration of hard or soft pines on the basis of the other. This knowledge is set forth in further detail in the following paragraphs.

9. Most classifications of *Pinus* recognize two major lineages: subgenus *Strobis* (haploxylon or soft pines, with one fibrovascular bundle in the needle) and subgenus *Pinus* (diploxylon or hard pines, with two fibrovascular bundles in the needle). This division is consistent with data from wood anatomy and secondary chemistry, and is supported in recent molecular phylogenetic studies. The genetic distance between subgenera, at least between *Pinus* and *Strobis*, may be as large as, or larger than the genetic distance between other conifer genera, e.g., between *Cedrus* and *Abies* (Price et al., 1987, *Systematic Botany*, 12:91-97 (copy attached as Exhibit 1)), and if strict genetic criteria were used, they should perhaps be treated at generic rank. As is commonly known, hard pines are unable to interbreed with soft pines, though they can interbreed readily, if the correct timing and other conditions are provided, with other hard pine species (a seminal reference is Critchfield and Little, 1966, *Geographic distribution of the pines of the world*, USDA Forest Service Miscellaneous Publication 991, Washington, D.C. (copy attached as Exhibit 2); see also Little and Critchfield, 1969, *Subdivision of the genus Pinus (Pines)*, USDA Forest Service Miscellaneous Publication 1144, Washington, D.C. (copy attached as Exhibit 3)). Hard pines are unaffected by a

number of diseases, such as white pine blister rust, that readily infect soft pines. Their susceptibility to *Agrobacterium* infection appears to be quite different as well (personal communications from Dr. Krystyna Klimaszewska and Dr. Armand Seguin, both of the Canadian Forest Service).

10. The differences between soft pines and hard pines have been shown for somatic embryogenesis of these pine subgenera. Specifically, Klimaszewska et al. (US 6,200,809 (copy attached as Exhibit 4)) demonstrates differences between soft and hard pines in the maturation of somatic embryos. Table 4 shows that number of somatic embryos and the germination percentage increased for soft pine (*Pinus strobus*) as the gellan gum content of the medium increased from 0.4% to 0.6% to 0.8% to 1.0%. Table 10, however, shows that the number of somatic embryos and germination percentage increased for hard pine (*Pinus taeda* (loblolly pine)) as the gellan gum content of the medium increased from 0.4% to 0.8% but decreased for hard pine (*Pinus taeda* (loblolly pine)) as the gellan gum content increased from 0.8% to 1.0%. The data in these tables further show that a higher concentration of ABA was used for the hard pine than was used for the soft pine and that the maximum germination achieved for hard pine was 57%, whereas the maximum germination achieved for soft pine was 92%. The knowledge and lack of expectation of success with respect to soft versus hard pines is also briefly described in the Declaration Under Rule 132 of Dr. Micahel Becwar filed in companion application Serial No. 09/973,089. (A copy of this Declaration is attached as Exhibit 5).

11. Prior to the present invention, there have been no reports of the regeneration of transgenic plants of pine of the genus *Pinus* subgenus *Pinus*. In fact any reports at all concerning regeneration of hard pines demonstrated that regeneration was not achieved. For example, Wenck et al. (1999, *Plant Mol Biol* 39:407-416; copy attached as Exhibit 6) specifically stated that stably transformed regenerated transgenic plants, of hard pine had not been obtained, although stably transformed regenerated transgenic plants of Norway spruce (*Picea abies*) had been obtained with *Agrobacterium* transformation. See page 413, bottom of left column for the statement concerning the lack of stably transformed regenerated transgenic plants for loblolly pine, a hard pine.

12. It is noteworthy that the cited Levee et al. reference did not discuss regeneration of transgenic plants of hard pine. Hard pines are the most economic species of conifers, and loblolly pine is the most used species of hard pines. Despite this fact, Levee et al. did not work with hard

pinus, but instead chose a species of soft pine. There have been no reports in the literature of the application of the method of Levee et al. to the regeneration of transgenic hard pines, either by Levee or by any other group. In addition, Levee has not continued use of the method with soft pines (personal communications from Dr. Krystyna Klimascewska and Dr. Robert Rutledge, both of the Canadian Forest Service). Finally, we have tried at Westvaco Corporation, the assignee of the present application, to use or modify for the regeneration of transgenic hard pine the method described by Levee et al. for the regeneration of a species of transgenic soft pine, but have not been successful. This lack of success with the Levee et al. method led to the present invention.

13. Experiments had been underway at Westvaco Corporation for more than 10 years to adapt systems for regenerating hard pines and for transforming and regenerating transformed hard pines. Somatic embryogenesis systems had been developed which worked well for regenerating hard pines. However, regenerating transgenic hard pines met with little or no success. Regeneration of transgenic hard pines produced by biolistic transformation procedures had been accomplished, but at only 2% efficiency. Regeneration of transgenic hard pines produced by *Agrobacterium* transformation had not been accomplished with six scientists working on the project. The inability to adapt systems developed for transgenic soft pines to transgenic hard pines is further evidence of the differences between soft pines and hard pines and the fact that there was no expectation of success in the art for using systems developed for transgenic soft pines in regenerating transgenic hard pines. This lack of success with the adaptation of other systems to regenerating transgenic hard pines led to the present invention.

14. In summary, a person of ordinary skill in the art at the time of the present invention knew that there were differences between soft pines and hard pines with respect to tissue culture, regeneration and transformation. In view of these differences and the lack of application of methods between the soft pines and the hard pines, there was no expectation of success in the art for regenerating transgenic hard pines on the basis of a single report for the regeneration of transgenic soft pines.

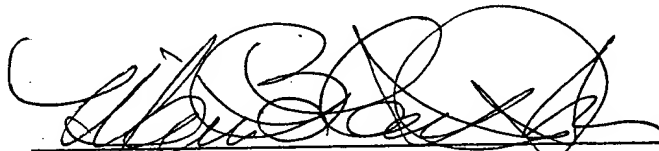
15. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

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Declaration Under Rule 132 of Marie B. Connett-Porceddu

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.

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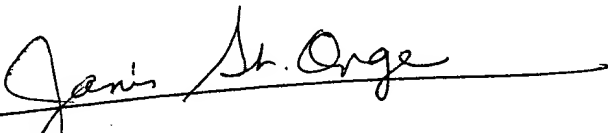
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Marie B. Connett-Porceddu

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Notary July 14, 2004

Dorchester County


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Relationships among the Genera of Pinaceae: An Immunological Comparison

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ABSTRACT. A radioimmunoassay comparison of seed proteins from nine of the ten genera of Pinaceae supports two major groupings in the family. These correspond to those of Van Tieghem, which are also supported by morphological evidence. The abietoid group, which forms a tighter cluster in the immunological analysis, consists of *Abies*, *Keteleeria*, *Cedrus*, *Tsuga*, and *Pseudolarix*, while the pinoid group consists of *Pinus*, *Picea*, *Cathaya*, *Larix*, and *Pseudotsuga*. Vierhapper's classification based on shoot dimorphism is thus rejected as artificial. *Pseudotsuga* and *Larix* form a well marked lineage within the pinoid group as indicated by both immunology and morphology.

The Pinaceae is the largest extant family of conifers, including ten genera and over 200 species (Sporne 1974). It is essentially restricted to the northern hemisphere and has apparently been so throughout its history (Florin 1963). Seven genera occur on two or more continents and have been widely studied. Less well known are *Cathaya*, *Keteleeria*, and *Pseudolarix*, small genera now restricted to portions of eastern Asia. The family is a natural one and is supported as monophyletic by its protein-type sieve cell plastids (Behnke 1974), pattern of proembryogeny (Dogra 1980), and lack of biflavonoids (Geiger and Quinn 1975). All of these features are unique to the Pinaceae among extant gymnosperms.

The history of the classification of the Pinaceae has been discussed by Florin (1931) and Flous (1936). Many groupings of the genera have been proposed, but two have been most widely used and will be emphasized here.

The classification of Van Tieghem (1891) divided the family into two "groups" (equivalent to subfamilies in the current system) based on the number and position of resin canals in the primary vascular region of the young taproot. The Myelocèles (=abietoid group) included *Tsuga*, *Cedrus*, *Abies*, *Keteleeria*, and *Pseudolarix*, with a single central canal, while the Epixylocèles (=pinoid group), included *Pinus*, *Picea*, *Pseudotsuga*, and *Larix*, with resin canals adjacent to each protoxylem pole. Jeffrey (1905) concurred in this classification based on com-

parative wood anatomy, while Doyle (1945) used it as the base for a diagram of the putative derivation of pollination types. *Cathaya*, described in 1958 by Chun and Kuang, is most similar to the members of the pinoid group in its wood anatomy and cone scale and seed morphology (cf. Chun and Kuang 1962; Greguss 1972) and was recently shown by Hu and Wang (1984) to have the pinoid type of root anatomy.

The second widely used classification is that of Vierhapper (1910), who divided the Pinaceae into two tribes based on occurrence and type of long shoot-short shoot dimorphism. Pineae included only *Pinus*, distinguished on the basis of its unusual short shoots (=needle clusters). Sapineae included the other eight known genera, and was further subdivided into two subtribes. Laricinae included genera with shoot dimorphism (*Pseudolarix*, *Larix*, and *Cedrus*), while Abietinae included the genera with only long shoots (*Abies*, *Keteleeria*, *Tsuga*, *Picea*, and *Pseudotsuga*). Florin (1963) recognized three subfamilies based on Vierhapper's groupings: Pinoideae (*Pinus*), Abietoideae (Vierhapper's Abietinae plus *Cathaya*, despite weak shoot dimorphism in the latter), and Laricoideae (Vierhapper's Laricinae), and these groupings were adopted by Krüssmann (1972).

More recently, Miller (1976, 1985) has emphasized the division between *Pinus* and the other nine genera based on consideration of certain features of ovulate cone anatomy among extant and fossil members of the family. Thus

Exhibit 1
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TABLE 1. Taxa and seed sources for immunological comparisons. The first six taxa fall into the abietoid group of Van Tieghem (1891) and the latter six into the pinoid group. Abbreviations for seed sources are as follows: Arnold Arboretum, Cambridge, MA (AA); D. P. Fowler (DF), cf. Prager et al. (1976); Inst. of Forest Genetics, Placerville, CA (PL); Maritime Forest Research Centre, Fredericton, New Brunswick (MF); Pacific Forest Research Centre, Victoria, B.C., (PF); Petawawa Forest Expt. Sta., Chalk River, Ontario (CR); Taiwan Forestry Res. Inst., Taipei (TF).

Taxon [Abbrev.]	Source
<i>Abies balsamea</i> (L.) Miller [AbB]	MF
<i>Cedrus deodara</i> (Roxb.) Loudon [CeD]	DF
<i>Keteleeria davidiana</i> (Franchet) Beissner var. <i>formosana</i> Hayata [KeD]	TF
<i>Pseudolarix amabilis</i> (J. Nelson) Rehder [PIA]	AA
<i>Tsuga heterophylla</i> (Raf.) Sarg. [TsH]	PF
<i>Tsuga mertensiana</i> (Bong.) Carrière [TsM]	PF
<i>Larix laricina</i> (Du Roi) K. Koch [LaL]	CR
<i>Picea abies</i> (L.) Karsten [PcA]	MF
<i>Picea rubens</i> Sarg. [PcR]	MF
<i>Pinus ponderosa</i> Lawson [PiP]	PL
<i>Pinus strobus</i> L. [PiS]	MF
<i>Pseudotsuga menziesii</i> (Mirbel) Franco [PtM]	PF

there has been considerable disagreement as to the relationships among genera in the Pinaceae based on their morphology.

Prager et al. (1976) conducted an immunological study of seed proteins in representatives of seven genera of the Pinaceae, using Ouchterlony double diffusion. They also used microcomplement fixation to obtain more precise data on a limited subset of the taxa. They were primarily interested in comparing rates of protein evolution and concluded that the seed proteins were changing at a rate comparable to that for intracellular proteins in higher animals, despite slower morphological evolution in the conifers. They did not directly address the question of suprageneric groupings in the family, but presented a Fitch-Margoliash tree consistent with Van Tieghem's classification.

In our current study we have applied a quite different immunological technique, the highly quantitative solid-phase radioimmunoassay (RIA), to corroborate the results of Prager et al. (1976) and explicitly test the classifications of Van Tieghem (1891) and Vierhapper (1910). RIA

has been previously applied to phylogenetic comparisons in a variety of animal groups (e.g., Lowenstein et al. 1981; Rainey et al. 1984) and in the chlorophycean algae (Olsen-Stojkovich et al. 1986). It is particularly valuable because it gives quantitative measures of immunological similarity using extremely small amounts of protein and is applicable to groups with a wide range of divergence times. In making phylogenetic inferences from the immunological data one assumes that the average rate of change over a large number of immunological determinants is sufficiently constant as to be approximately proportional to divergence time. This allows an assessment of relationships independent of that from morphology.

In this study we have included representatives of all five of Van Tieghem's abietoid genera and four of the five pinoid genera. Seeds of *Cathaya* are currently unavailable outside of mainland China. The position of the monotypic East Asian genus *Pseudolarix* is of particular interest in that it is placed very differently in the two classifications under consideration. We have also examined and re-analyzed available morphological data for comparison to the immunological results.

MATERIALS AND METHODS

Taxa and seed sources used in the immunological comparisons are listed in table 1. Fully matured germinable seeds of *Pseudolarix amabilis* and *Keteleeria davidiana* were decoated, homogenized, and extracted in isotris buffer (Champion et al. 1974) for four days under rotation. Antisera were prepared using one ml aliquots of the protein-rich supernatant extract injected into New Zealand white rabbits according to the following schedule: primary immunization in Freund's complete adjuvant followed by three secondary injections at two to three week intervals with bleeding at twelve weeks. Antisera and seed extracts for the other taxa were obtained from Prager and Wilson (cf. Prager et al. 1976). All antisera and seed extracts were stored frozen at -10°C .

A "sandwich-technique" RIA (Tsu and Herzenberg 1980; Lowenstein et al. 1981) was used to measure immunological differences among taxa. Because antisera vary in their strength and titer, scout titration assays of homologous reaction pairs were performed in order to deter-

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TABLE 2. Cross-reactions of conifer seed proteins. Units are percent uptake of labelled GARGG, with results standardized by rows to give homologous reactions of 10. Rows show reactions to a given antibody and columns to a given antigen. See table 1 for names of taxa.

Anti-body	Antigen											
	KeD	PIA	PIP	PIs	CeD	TsM	TsH	PcR	PcA	LaL	AbB	PtM
KeD	10.0	6.26	4.11	3.68	6.67	5.41	7.69	3.08	2.84	5.47	7.94	4.32
PIA	4.70	10.0	3.57	1.90	3.87	5.33	5.95	3.72	2.53	3.49	4.17	2.17
PIP	3.49	4.07	10.0	8.84	4.44	6.44	3.75	5.74	5.16	6.29	6.23	5.11
PIs	5.04	2.16	4.30	10.0	1.80	2.50	1.63	2.04	2.40	2.50	2.32	3.43
CeD	7.51	5.77	5.46	6.35	10.0	8.04	5.89	4.18	4.59	6.57	10.0	7.50
TsM	4.56	5.16	2.60	3.02	3.36	10.0	5.30	2.98	2.72	4.00	5.46	3.64
TsH	3.26	7.17	4.27	3.60	4.43	9.44	10.0	2.32	2.38	3.51	5.48	4.11
PcR	6.20	4.17	6.73	6.13	3.93	4.67	4.03	10.0	10.0	4.40	4.53	3.60
PcA	4.53	4.70	4.36	4.76	3.20	4.38	2.78	8.32	10.0	4.42	3.81	3.37
LaL	5.57	1.15	5.08	4.59	1.10	4.85	2.27	1.57	1.54	10.0	3.51	5.82
AbB	6.75	2.63	2.33	2.61	3.61	4.19	4.02	1.71	1.71	2.02	10.0	3.38
PtM	4.07	1.56	3.18	3.51	1.82	2.95	1.76	3.68	4.21	6.62	2.78	10.0

mine appropriate reaction concentrations. Optimum antibody concentration is taken from that linear portion of the reaction curve yielding the highest percent binding. Because the antigen phase is bound to the plate, changes in its concentration have less effect. By comparison of a series of homologous reaction curves among different taxa, appropriate dilution adjustments can be made to insure heterologous comparisons are made from the same area of the curves. Both the antigen and homologous antiserum were used for all taxa examined, and all possible antigen-antibody combinations were produced. The resulting immunological distances were standardized using the bindings from the reciprocal homologous reactions.

In the RIA assay, 20 μ l of appropriately diluted antigen were bound to each well in polyvinyl microtiter plates (Dynatech Laboratories). After a 1-hr incubation, the unbound material was washed from the plate with 2% bovine serum albumin (BSA), which also blocks further binding to the plate. Twenty μ l of appropriately diluted antiserum were then added to each well and allowed to react for 24 hr at room temperature. Unreacted antibody was removed by suction followed by three 2% BSA washes. Finally, 20 μ l of a second marker antibody [125 I-labelled goat anti-rabbit-gammaglobulin (GARGG)] were added to each well and allowed to incubate for an additional 24 hours. The amount of GARGG bound is proportional to antibody bound in the second step. Excess GARGG was washed out with ten dis-

tilled water rinses. The plates were sealed with plastic tape, cut up, and counted for radioactivity on an LKB Gamma Counter.

Immunological distance (ID) was calculated using $ID = -100 \log$ Immunological similarity (IS). IS is equal to the sum of the bindings for the reciprocal heterologous reactions divided by the sum for the homologous reactions. Trees were constructed according to the Fitch and Margoliash (1967) algorithm using program FITCH in the program package PHYLIP (version 2.1), written by Joseph Felsenstein (University of Washington, Seattle) and by UPGMA clustering (Sneath and Sokal 1973).

RESULTS

The matrix of binding values for cross-reactions among taxa of Pinaceae is presented in table 2 and the resulting immunological distance matrix in table 3. The values in table 2 are the averaged percent binding of two to three replicate experiments. Reciprocity of cross-reactions (i.e., equivalence of binding values for antigen A vs. antibody B and vice versa) is not always good, but averaging of the reciprocal values tends to compensate for this. Low reciprocity results from complexity of immunological systems, in which antisera and antigens differ in strength and each antiserum reacts with a somewhat different group of antigenic sites. Results of our RIA technique on other study groups (e.g., genera of chlorophycean algae; Olsen-Stojkovich et al. 1986) indicate that rep-

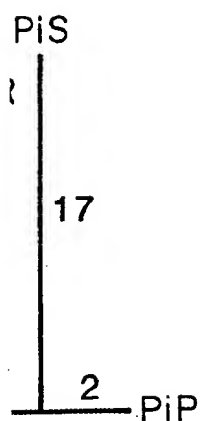
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METHODS

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in the cluster dial distance matrix) analysis of dis- v and column-col- aw bindings (table opology to that in ects of reciprocity tree based on the shown in figure 2. Tieghem's abietoid

LaL	AbB	PtM
26	13	38
63	47	73
24	37	38
45	61	46
42	17	33
35	32	48
54	32	53
52	51	44
53	56	42
0	56	21
	0	51
		0

ABIETOID GROUP

PINOID GROUP

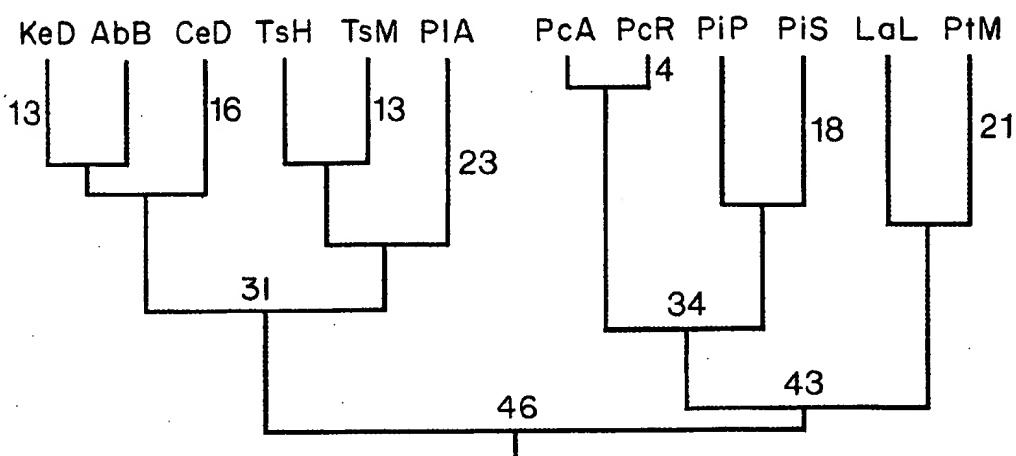


FIG. 2. UPGMA tree of immunological distances among taxa of Pinaceae. See table 1 for names of taxa.

group are placed together in one compact group in our figures 1 and 2, while the four genera examined from the pinoid group are more loosely associated. *Larix* and *Pseudotsuga* are placed on their own lineage within the pinoid group. In our analyses, *Abies*, *Cedrus*, and *Keteleeria* were consistently placed together, but there is some uncertainty as to the order of branching among them. Unsuccessful attempts were made to resolve the trichotomy using absorbed antisera (see Lowenstein et al. 1981 for details of competitive inhibition techniques).

Pseudolarix consistently showed a strong reaction with the *Tsuga* species, but was positioned somewhat differently on the two tree diagrams. In the UPGMA tree (fig. 2), which is based on a more local averaging of distances, the two species of *Tsuga* (representing the two subgenera) are shown as a distinct group adjacent to *Pseudolarix*, while in the FITCH tree (fig. 1), *Pseudolarix* is placed terminal to *Tsuga*. The two species of *Tsuga* are more similar immunologically to one another (ID = 13) than either is to *Pseudolarix* (ID = 18 and 28), so the positioning on the FITCH tree seems less appropriate.

The three pairs of congeneric species included in our analyses provide an internal check on the reliability of the immunological distances. The two species of *Picea*, from the same subgenus, behave almost identically in the im-

munological comparisons (ID = 4), while the pairs of species from different subgenera of *Pinus* and *Tsuga* are appropriately more distinct (ID = 18 and 13, respectively).

DISCUSSION

Our immunological results place *Pseudolarix* and *Keteleeria* in the abietoid group and thus concur exactly with Van Tieghem's groupings. In contrast, the genera of Vierhapper's Laricinae (*Larix*, *Pseudolarix*, and *Cedrus*) are not shown to be similar immunologically to one another, implying multiple origins and/or losses of shoot dimorphism.

A number of morphological features also support the groupings of Van Tieghem. In addition to the difference in root anatomy noted by Van Tieghem (1891), the five pinoid genera have "normal" resin canals in both the vertical and horizontal systems of their stem wood (Phillips 1948; Hu and Wang 1984), while the abietoid genera have only traumatic resin canals in the stem wood with the apparent exception of the vertical system in *Keteleeria*. The abietoid genera all have seed coats containing resin vesicles, while these are lacking in the pinoid genera (Hickel 1911; Chun and Kuang 1962; confirmed on fresh material by the current authors except for *Cathaya*). It appears that these characters are not logically or function-

ally dependent on one another, and their distribution is quite unlikely to have occurred by chance. Preliminary cladistic analyses of morphological and anatomical features of the Pinaceae (Price et al., unpubl. data) indicate that the abietoid and pinoid groups of Van Tieghem are at least convex (i.e., either both are monophyletic or one gave rise to the other and is thus paraphyletic), while the Laricinae and Abietinae of Vierhapper are polyphyletic.

Within the pinoid group, *Larix* and *Pseudotsuga* are supported as sister groups by our immunological results and those of Prager et al. (1976). Despite their dissimilarity due to the winter-deciduous habit and marked shoot dimorphism of *Larix*, it has long been known that they share a number of morphological features. Both genera have an unusual type of nonsaccate pollen unique among conifers (Erdtman 1965) and a specialized type of micropylar apparatus at the time of pollination (Doyle 1945). Both also have clusters of "fiber-sclereids" in their bark (Chang 1954; Srivastava 1963). All of these features are unique to these two genera within the Pinaceae and are evidently derived features based on outgroup comparison. The two genera also share an unusual karyotype of six isobrachial and six markedly heterobrachial and smaller chromosomes (Khoshoo 1962; Simak 1966; El-Kassaby et al. 1983—*Pseudotsuga menziesii* has an aneuploid derivative of this karyotype with $n = 13$) and have similar seeds and cone scales. Thus the close relationship of these two genera seen in the immunological analysis is well supported by morphological evidence.

The three subgroups seen within the pinoid group (*Pinus*, *Picea*, and *Larix* plus *Pseudotsuga*) are more distant from one another in our tree diagrams than the genera within the abietoid group. This reflects the average of a number of separate distances and presumably is due to a greater time of divergence. *Pinus* has the longest well established fossil record of the extant genera, dating back to the early Cretaceous (Miller 1976). It is also distinct in a number of characters (e.g., cone scale form and anatomy, and possession of specialized needle clusters). Miller has suggested a major split between *Pinus* and the other extant genera, which would lead to a prediction that *Pinus* should be more distant immunologically from the other genera than they are from one another. This was not observed in our results or those of Prager et al.

(1976), which seem to indicate considerable age for *Picea* as well. Further studies with a more diverse sampling of taxa and other macromolecular techniques would be valuable in addressing the question.

Within the abietoid group, *Abies*, *Keteleeria*, and *Cedrus* are approximately equidistant and quite similar to one another based on the immunological data. Their morphological differences are important enough, however, that they definitely should be retained as separate genera despite their immunological similarity. *Keteleeria* has generally been considered to be most similar to *Abies* or *Pseudolarix* in its morphology (de Ferré and Gaussen 1945; Dallimore and Jackson 1966). It has leaf scars flush to the stem as in *Abies*, umbellate arrangement of male cones as in *Pseudolarix*, and similarities of cone scale and seed morphology to both. Our immunological analysis places *Keteleeria* closest to *Abies* and *Pseudolarix* closest to *Tsuga*. The latter result is unexpected on the basis of morphology and is in need of independent confirmation.

Cedrus has generally been considered most similar to *Abies* based on its ovulate cones, which consist of tightly packed, broadly fan-shaped scales that abscise from the axis at maturity. This accords with their immunological similarity. They also differ in a number of features, however, with *Cedrus* having shoot dimorphism, regular occurrence of ray tracheids in the wood, and broader attachment of the pollen saccae (Phillips 1948; Sivak 1975).

In conclusion, analyses of seed proteins using radioimmunoassay yield groupings identical to those of Van Tieghem (1891), placing *Abies*, *Keteleeria*, *Cedrus*, *Tsuga*, and *Pseudolarix* in one group and *Pinus*, *Picea*, *Larix*, and *Pseudotsuga* in another, to which *Cathaya* would be added on the basis of its morphology. Our results using radioimmunoassay are consistent with those obtained from Ouchterlony double diffusion by Prager et al. (1976) and provide a useful check on the methods. Groupings from our immunological trees are concordant with a number of morphological characters and provide a framework for more detailed phylogenetic analysis.

ACKNOWLEDGMENTS. We thank Ellen Prager and Allan Wilson for providing antisera and seed extracts from their previous study and Pao-chang Kuo, David Hu, and the staff of the Arnold Arboretum for pro-

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viding seed samples. Gary Scheuenstuhl performed our immunological assays. William Critchfield, Charles Miller, and Robert Ornduff made useful comments on the manuscript.

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*Exhibit 2
Connett '08*

Subdivisions of the
Genus *PINUS* (Pines)

by

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Miscellaneous Publication No. 1144

Exhibit 3
Connett '088

Library of Congress Catalog Card Number: Agr 70-602503.

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Subdivisions of the Genus *Pinus* (Pines)

By Elbert L. Little, Jr., and William B. Critchfield¹

INTRODUCTION

A summary of the nomenclature and 22 distribution maps of subdivisions of the genus *Pinus* L., pine, are presented here to supplement Miscellaneous Publication 991—Geographic Distribution of the Pines of the World, with maps of 94 species (Critchfield and Little 1966). Important synonyms and lists of the species are included, and the slight changes in the classification of the subdivisions are explained. The maps of subdivisions combined from those of individual species show clearly and graphically the maximum natural range of all component species. These compiled maps of groups of related species may be useful in further researches, particularly in classification, geographical distribution, tree breeding and introduction, and evolution.

In recent years several revised classifications of the genus *Pinus* have been proposed, some emphasizing anatomy of needles (Jahrig 1962), of wood (Hudson 1960), and of cotyledons (Ferré 1965). Others, such as those by Ishii (1952) and Gausson (1960), represent new interpretations based upon various, mostly morphological characters. Numerous interspecific hybridization tests at the Institute of Forest Genetics, Placerville, Calif., and elsewhere, have suggested modifications of the classification according to crossability. The slightly revised classification adopted here incorporates these changes.

A check of the nomenclature of the subdivisions of the genus *Pinus* is needed to determine the correct names under the International Code of Botanical Nomenclature (Lanjouw 1966). These mostly retroactive rules have been changed since some names were published, and some recently published names are not in accordance with the Code. Some problems in determining the correct section names in a large genus have been noted recently by Burtt (1966). A few older classifications and names have been overlooked or passed over by other workers. The earlier subdivision names were not indexed or covered by rules. Naturally, many names published for remodelled groups were superfluous. A few arrangements were merely convenient groups of the species in-

cluded in a publication, somewhat like the keys, and not intended as revisions of nomenclature of the genus. The number of ranks between genus and species in the genus *Pinus* has varied in time and among authors. When the rank of a taxon is changed, competition for priority begins anew.

In an effort to bring the nomenclature up to date, we have compiled the significant synonymy, chosen a few lectotype species as needed, and have emended or altered some groups in diagnostic characters and in circumscription. Names established in usage have been retained so far as possible.

Slight changes in the classification of Shaw (1914, 1924) were adopted in the recent publication (Critchfield and Little 1966), and an explanation is offered here. The three ranks of subdivisions recognized here within the genus *Pinus* are subgenus, section, and subsection. A separate subgenus (with section and subsection) is accepted for *Pinus krempfii* Lecomte, as Ferré (1953, 1965) and Gausson (1960) proposed. The important revision by Duffield (1952) of *Pinus* subsection *Pinaster* has been followed except for his union of one small group. The needed scientific names have been assigned to Duffield's four groups XI–XIV, which were designated merely by roman numerals. Accordingly, we proposed three new names of subsections, *Pinus* subsect. *Krempfianae*, subsect. *Contortae*, and subsect. *Oocarpae*. Also, we validly published the combination *Pinus* subgen. *Ducampopinus* (A. Cheval.) de Ferré (1953) with full reference to original publication. *Pinus* subsect. *Pineae* is validly published here.

REVIEW OF INTERNATIONAL CODE OF BOTANICAL NOMENCLATURE

The more important Articles in the International Code of Botanical Nomenclature (Lanjouw 1966) relating to nomenclature of subdivisions of generic names are Articles 4, 10, 21, 22, 33–37, 51, 60, 63, and 66. A brief review may be appropriate here.

The ranks of subdivisions of genera (ICBN Art. 54, footnote) are: Subgenus, sectio, subsectio, series, subseries (Arts. 4, 21), and the types are species (Art. 10). A name or an epithet does not have priority outside its own rank (Art. 60). An alteration of the diagnostic characters or of the circumscription does not warrant a change in names, with certain exceptions (Art. 51). An epithet

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must be rejected if its author did not adopt the earliest legitimate epithet available for the taxon with its particular circumscription, position, and rank (Art. 66). A name must be rejected if it was nomenclaturally superfluous when published, if the taxon as circumscribed by its author included the type of a name or epithet which ought to have been adopted (Art. 63). The subgenus or section including the type species repeats the generic name as its epithet but without the author's name, and a section including the type species of any subgenus must bear as its epithet the epithet of the subgenus (Art. 22). The epithet of a subgenus or section is preferably a substantive, that of a subsection or lower subdivision of a genus preferably a plural adjective (Art. 21, Rec. 21B). When the epithet is identical with or derived from the epithet of a constituent species, this species is the type (Art. 22, new paragraph added in 1966).

A few pertinent Articles are not retroactive. Names published after Jan. 1, 1935, must be accompanied by a Latin diagnosis or reference to a previously published Latin diagnosis (Art. 36). Names published after Jan. 1, 1958, are valid only when the nomenclatural type is indicated (Art. 37). A new combination published after Jan. 1, 1953, must indicate clearly the basionym with a full reference to its author and original publication (Art. 33). A new name published on or after Jan. 1, 1953, without a clear indication of the rank of the taxon concerned is not validly published, but for such names published earlier, the choice made by the first author who assigned a definite rank must be followed (Art. 35 and Note). The rank "group" (also "Gruppe," "groupe," and "grupo") is not mentioned (Art. 4). However, such names published before Jan. 1, 1953, have the first rank assigned by a later author (Art. 35, Note).

Some recently published names of subdivisions of the genus *Pinus* are superfluous and illegitimate because the taxa containing the type species have older names (Arts. 51, 63). If the genus *Pinus* should be divided, the name *Pinus* must be retained for one of the segregates, and the same general rule applies to remodelling subdivisions of genera (Arts. 51, 52). If a subdivision is remodelled by transfer of some species to or from it, the name must be retained for the taxon containing the type species. Fortunately, the holotype of most epithets was clearly designated by the derivation from the epithet of a species (Art. 7). When a section is divided, there may be available an earlier epithet of different circumscription but with the same type species. A second epithet of the same rank based upon the same type species is illegitimate, for example, *Pinus* sect. *Banksianoides* later than sect. *Banksia* (Arts. 63, 66). Likewise, a new classification of the genus *Pinus* with all epithets of sections new or with new endings must be rejected. Examples are the 10 sections proposed by Gaussen (1960) and the five sections of Hudson (1960). These last and some other recently published names must be rejected also because they were not accompanied by a Latin diagnosis.

THE GENUS *PINUS* AND ITS CIRCUMSCRIPTION

Pinus [Tourn.] L. is a classical Latin name. Tidestrom (Elys. Marian. Evergr. 72. 1908) cited Pliny as author. Lunell (Amer. Midland Nat. 4: 160. 1915) credited the authorship to Virgil (Ecl. VIII: 56; Georgica I: 141). *Pinus* L. (Sp. Pl. 1000. 1753; Gen. Pl. ed. 5, 434. 1754) originally contained 10 species: *sylvestris*, *Pinea*,

Taeda, *Cembra*, *Strobus*, *Cedrus*, *Larix*, *Picea*, *Balsamea*, and *Abies*. This genus united 3 pre-Linnaean genera *Pinus*, *Abies*, and *Larix* of Tournefort (Elem. Bot. 1: 457, t. 355-356. 1694). Earlier: Linnaeus (Gen. Pl. ed. 1, 731. 1737; ed. 4, 357. 1752) had followed Tournefort's narrower circumscription of *Pinus*. The lectotypus, *Pinus sylvestris* L., was selected by Britton and Shafer (N. Amer. Trees 5. 1908) and by Hitchcock and Green (Internat. Rules Bot. Nomencl. Brittonia 6: 117. 1947).

The genus was promptly remodeled and emended by Miller (Gard. Dict. Abridged. Ed. 4. 1754) who, while merely following Tournefort and not citing Linnaeus, segregated *Abies* and *Larix*. Later segregates were *Cedrus* Trew (Cedrorum Libani Hist. 4. 1757) and *Picea* A. Dietr. (Fl. Berlin 794. 1824). However, after more than a century, Parlatores (in DC., Prodr. 16(2): 377-407. 1868) still used the broad, original circumscription of *Pinus* L. *sens. lat.* Nearly all contemporaneous specialists accept *Pinus sens. strict.* as emended by Miller. A few later generic segregates have also been proposed, as indicated in the synonymy, but have received very limited acceptance. Briefly, the genus *Pinus*, as almost universally defined, is distinguished from all other conifers by the needle-like leaves on dwarf shoots in fascicles of usually 2-5 with a sheath of bud-scales at base.

PRINCIPAL CLASSIFICATIONS OF THE GENUS *PINUS*

A review of the principal classifications of subdivisions of the genus *Pinus* in chronological order will indicate the available names and serve as a basis for determining the correct nomenclature. Compilations of names of subdivisions in the genus *Pinus* were made by Pfeiffer (Nomencl. Bot. 2: 722-723. 1874) and by Rehder (Bibliog. Cult. Trees Shrubs 32-41. 1949). The first ended with the year 1858. Rehder's Bibliography contains the most detailed synonymy available but covers only cultivated trees hardly in the cooler temperate regions and omits pines of warm temperate and tropical regions.

The first division of the genus *Pinus* after 1753 was by Duhamel (Traité Arb. Arbust. France 2: 124-126. 1755). His three "sections," *Bifolius*, *Trifolius*, and *Quinquefolius*, based upon number of needles in a fascicle or sheath, had brief French descriptions and appeared in both text and headings. These epithets not cited by later authors were validly published, even though the Linnaean system of binary nomenclature was not employed and the endings were in ablative case. Duhamel's first and third epithets are replaced automatically by those repeating the subgenera *Pinus* and *Strobus* (ICBN Art. 22). His second section can be rejected by union with the first.

Probably the first subdivision of *Pinus* L. *sens. lat.* to correspond to *Pinus* L. *sens. strict.* and repeat the generic name was *Pinus* I. *Pinus* Münchhausen (Hausvater 5: 215. 1770). David Don (Prodr. Fl. Nepal. 54. 1825) apparently was the first to propose *Pinus* [group] *Strobus* for a group of undesignated rank containing two species. Pfeiffer (Nomencl. Bot. 2: 722, 1303. 1874) designated the rank as section. This epithet is often credited to Sweet (Hort. Brit. ed. 2, 475. 1830), whose subdivisions in a table of species were *nomina nuda* without descriptions or indications of rank (Art. 34).

Loudon (Arb. Frut. Brit. 4: 2152-2292, illus. 1838), in a detailed classification overlooked by some later authors, divided the

genus into three sections, Sect. *Binae*, *Ternatae*, and *Quinae*. The first and third are now replaced by the epithets *Pinus* and *Strobus*. Sect. *Ternatae* is typified here to be a synonym of Sect. *Pinus*. Loudon introduced a second rank with 15 groups derived from specific epithets, designated by the symbol §, and described under "Sect. Char." Examples are *Sylvestres*, *Taeda*, *Australes*, *Cembrae*, *Strobi*. As the symbol § generally means section, this rank below section corresponded to subsection.

Spach (Hist. Nat. Végét. Phan. 11: 369-403. 1842) divided the genus *Pinus* into 4 sections, *Eupitys*, *Taeda*, *Strobus* Sweet, and *Cembra*.

Endlicher (Syn. Conif. 81-183. 1847) in synonymy used the term subgenus, possibly for the first time in *Pinus*. He divided *Pinus* L. sens. lat. into two groups *Sapinus* and *Pinus* corresponding to subgenera and further into 11 sections ("sectio"), six of these within *Pinus* sens. strict.

Carrière (Traité Gén. Conif. 291-412. 1855; ed. 2, 381-589. 1867) substituted the rank tribe ("tribu") for section (contrary to Arts 4, 5, 33).

Gordon and Glendinning (Pinetum 162-267. 1858; ed. 2, 228-326. 1875) distinguished only the three sections proposed by Loudon, *Binae*, *Ternatae*, and *Quinae*.

Parlatore (in DC., Prodr. 16(2): 377-407. 1868) modified Endlicher's classification slightly. He recognized within *Pinus* L. sens. lat. subgenus *Pinus* the two sections ("sectio") *Pinea* and *Cembra*, the former subdivided into three groups of undesignated rank reduced from section, *Pinaster*, *Taeda*, and *Pseudo-Strobus*.

Koch (Dendrologie 2 (2): 269-325. 1873) divided *Pinus* into 6 groups ("Gruppe"), *Sabinea* being new.

Engelmann (St. Louis Acad. Sci. Trans. 4: 161-189. illus. 1880) in a brief key to the species accepted two sections *Strobus* and *Pinaster* and eight subdivisions designated by §. The six subdivisions under sect. *Pinaster* were further subdivided into 14 named groups of species and two unnamed groups of one species each. These subdivisions were called "subsections" (p. 175) and in the notes "subsection *Cembroides*" (p. 178), "*Australes* group" (p. 183, § on p. 177) and "*Pseudo-Strobi* group" (p. 185).

Bentham and Hooker (Gen. Pl. 3(1): 438-439. 1880) accepted only Engelmann's two primary sections but noted that Engelmann in correspondence had proposed 20 subsections. Eichler (in Engler & Prantl, Natürl. Pflanzenfam. 2(1): 70-74. 1889) distinguished the same two sections and five groups of a lower rank designated by §. Sargent (Silva No. Amer. 11: 4. 1897) accepted and further defined Engelmann's two sections and eight groups of lower rank.

Mayr (Wald. Nordamer. 425-428. 1890; Fremdl. Wald. Parkbaume 340-390. 1906) developed a natural classification with 11 sections, six with new names.

Koehne (Deutsche Dendrologie 28-40. 1893) proposed the widely accepted division of the genus into two groups based upon the presence of one or two vascular bundles in the leaf or needle, Sekt. *Haploxyton* and Sekt. *Diploxyton*. These appropriately descriptive names of correct circumscription obviously lack priority and are illegitimate as section names under present rules, not repeating the subgeneric epithets, also not available under rank of subgenus. These sections were subdivided into six subsections ("Subsekt.") and the first two subsections also into two groups each ("Gruppe").

Lemmon (Handb. West-Amer. Cone-bearers ed. 3, 19-46. 1895) published a detailed, overlooked classification of *Pinus* in Western North America with four ranks: Subgenus, section, subsection, and "group." Apparently he was the first to use the rank subgenus for the two main subdivisions designated as sections by Engelmann, *Strobus* for softwood or white pines and *Pinaster* for hardwood pines. The first was subdivided into two named groups, and the second into two sections, two subsections, and seven groups. *Pinus* subgen. *Strobus* Lemm. is accepted here.

Masters (Linn. Soc. London J. Bot. 35: 560-659. illus. 1904) in a key grouped the species into divisions *Tenuisquamae* and *Crassisquamae* and further into 10 sections, five of them new.

Silva Tarouca (Unsere Freiland Nadelhölzer 235-265. 1913) adopted the rank "Hauptgruppe" for *Haploxyton* and *Diploxyton* and "Gruppe" for the nine subdivisions. In a later edition, Silva Tarouca and Schneider (Unsere Freiland-Nadelhölzer ed. 2, 240-271. 1923) followed Shaw (1914) but retained the same rank names and called the third rank "Reihe," corresponding to series.

In the classic monograph, Shaw (1914) distinguished two sections (*Haploxyton* and *Diploxyton*), four subsections (*Parapinaster* proposed as new), and 13 "groups" designated by name and roman number, partly taken from Engelmann and five new. Afterwards, Shaw (1924) united his group *Flexiles* with group *Strobi* and placed the newly discovered anomalous species *Pinus krempfii* Lecomte under group *Balfourianae*.

Apparently the first to elevate Koehne's two sections *Haploxyton* and *Diploxyton* to subgenera was Rehder (in Bailey, 1923, pp. 295-331), who should be credited as author. However, in a later publication, Rehder (Man. Cult. Trees Shrubs 53-66. 1927) cited Koehne. He raised Shaw's four subsections to sections but left Shaw's "groups" as such.

The transfers of *Haploxyton* and *Diploxyton* to subgenera generally have been credited to Pilger (in Engler & Prantl, Natürl. Pflanzenfam. ed. 2, 13: 331-342. illus. 1926) rather than Rehder. Besides these two subgenera ("Untergatt.") Pilger distinguished 11 sections ("Sekt.") and two subsections ("Untersekt."). Fitschen (Beissner Handb. Nadelholzk. ed. 3: 326-433. 1930) modified Pilger's classification slightly, accepting nine sections and five groups ("Gruppe"). Melchior and Werdermann (Engler's Syllabus Pflanzenfam. ed. 12, 1: 331-332. 1954) followed Pilger with omission of the two subsections. Komarov (Fl. URSS. 1: 159-173. 1934) also accepted Koehne's two subdivisions as subgenera (podrod) and had five sections (sektiia).

The requirement of Latin diagnosis for names of new groups published after Jan. 1, 1935 (Art. 36), results in rejection of many later names. Several later classifications merit mention, though the new names mostly are not in accord with the Code.

The rank series was first used in the genus *Pinus* apparently by Rehder (1940, pp. 34-40). He changed Shaw's groups to series but they were without author or Latin diagnosis. Afterwards Rehder (1949, pp. 32-41) made slight changes in names of several groups, proposed new combinations for the two subgenera *Strobus* (Sweet) Rehd. and *Eupitys* (Spach) Rehd., and adopted section and series for the two lower ranks. Krüssmann (Nadelgehölze 206-236. 1955; ed. 2, 220-257. 1960) followed Rehder.

Martínez (1945) arranged the Mexican pines into nine sections ("Sección") and six groups ("grupo") but without Latin diagnoses.

Mme. van Campo-Duplan (1950, p. 92, 94) in classifying the species of *Pinus* according to their pollen grains used for subgenera ("sous-genres") the names *Haplopinus* and *Diplopinus* but without description there.

Mlle. Yvonne de Ferré (1953) proposed with French descriptions four subgenera ("sous-genres") as follows: *Diplopinus* van Campo (*Diploxylon* Koehne), *Cembra* de Ferré (*Cembra* Koehne), *Paracembra* de Ferré (*Paracembra* Koehne), and *Ducampopinus* (A. Cheval.) de Ferré. The last, a new combination, did not cite the page of basionym.

Duffield (1952) made a major rearrangement of Shaw's classification of Subsection *Pinaster* on the basis of results of interspecific hybridization, designating four groups by roman numerals (XI–XIV) without scientific names.

Ishii (1952) proposed two new subgeneric names, Subgenus *Malacopitys* for the soft pines and Subgenus *Scleropitys* for the hard pines, both without Latin diagnoses. In a Japanese key to 82 species, he distinguished 13 sections, four of these new and without Latin diagnoses.

The most detailed recent classification was by Gausson (1960), who did not supply Latin diagnoses. He accepted three subgenera ("sous-genre"), *Ducampopinus*, *Eupinus*, and *Cembra*. Also his classification (p. 38) contained 10 sections with new names ending in *-oides* (*Kremfoides*, *Taetoponderosoides*, etc.) and 33 groups ("group") designated merely by specific epithets without Latin diagnoses.

Mirov (1961, p. 27; 1967) followed Shaw but rearranged the species and groups slightly according to composition of gum turpentine.

Jährig (1962) proposed a classification based mainly upon needle anatomy with a key to species. The two subgenera ("Untergattung") *Pinus* and *Haploxylon* were subdivided into 11 sections ("Sectio"), eight from Gausson (1960) and three new but without Latin diagnoses.

Debazac (1964, pp. 82–112) accepted the three subgenera ("sous-genre") adopted here, *Pinus*, *Strobilus*, and *Ducampopinus*, also eight sections and eight subsections ("sous-section").

Classifications based on wood anatomy have been relatively simple and composed of few groups. Phillips (1941) accepted the seven groups recognized by R. Rol in 1932. Greguss and Varga (1950) had nine groups ("Gruppe"). R. H. Hudson (1960) divided the genus *Pinus* into only five sections (without Latin diagnoses): *Cembra*, *Paracembra*, *Parapinaster*, *Pinaster-Laricoides*, and *Pinaster-Taeda*.

RANKS IN THE GENUS *PINUS*

Obviously, the nomenclature adopted for the subdivisions of the genus *Pinus* is dependent upon the number of ranks distinguished and their names. The three ranks accepted here are subgenus, section, and subsection.

Duhamel (1755) introduced section. Loudon (1838) had a second rank §, under which the description was headed "Sect. Char." and thus a second rank of section, or subsection. Engelmann (1880) distinguished three ranks, section and two ranks of subsections, the first designated by §.

Lemmon (1895) had four ranks: subgenus, section, subsection, and group. Apparently he was the first to use the rank subgenus for subdivisions within the present genus *Pinus* L. sens. strict.

Rehder (1940, 1949) introduced the rank series. His three ranks were subgenus, section, and series.

At present the species of *Pinus* (94 accepted here) are grouped more or less naturally and conveniently into three ranks. The highest rank, the subgenus, contains only three examples of major taxonomic groups, such as the soft pines and hard pines. The second rank, section, has only five examples. For the third rank, subsection with 15 examples clearly has priority over series and much wider usage and is adopted here. Continued use of subsection will result in fewer name changes than adoption of the relatively new rank of series.

Group is rejected as a rank and should not be used with Latin epithets (Art. 4). If a simpler classification of two ranks is desired, the names are subgenus and section. Or, if a simplified classification of several groups in one rank should be desired, the logical name is section. Of course, the names and epithets of the 15 subsections can be used in this way with the generic name or alone, without mention of their subgenera and sections. Otherwise, some epithets of subsections would be changed if the same groups were treated as sections.

The problem is selection of epithets for the 15 taxonomic groups with rank of subsection. The earliest names with rank below section are the 15 taxonomic groups of Loudon (1838) designated by § and "Sect. Char." These epithets have priority, and their type species are indicated by their derivation (Art. 22). Pilger (1926) cited Loudon's names, though some later authors did not. Some of Loudon's epithets derived from those of species were proposed independently by later authors. Several additional names with rank below section are among the subsections, eight designated by § and 14 of lower rank, distinguished by Engelmann (1880).

Thus, the genus *Pinus* may be subdivided into 15 groups of rank subsection, for example, *Pinus* subsect. *Australes*. Table 1 compares the nomenclature adopted here with that of four other classifications of the genus *Pinus*. Names of nine of the 15 subsections are credited to Loudon and two to Engelmann. We have supplied from specific epithets the names needed for the remaining four small subsections, two with only one species each.

SUMMARY OF SUBDIVISIONS OF THE GENUS *PINUS*

The revised nomenclature of the subdivisions of the genus *Pinus* is summarized below. Each accepted name is followed by its citation, basionym if any, type species (holotype or lectotype), pertinent synonyms in use for the same and related ranks including "group" of Shaw and citation by Rehder as series, number and list of included species, with citation, English common name, and geographical distribution of each. Brief descriptions of the taxonomic groups have been adapted and emended from Shaw. Only the main morphological characters are mentioned. Other characters, such as chemistry, have not been included. Latin diagnoses of the subdivisions have been added for completeness, though not required under the Code. Some of these names published long ago have been emended by later authors.

The synonymy is not intended to be complete. It indicates the ranks assigned by later authors to groups published without clear indication of rank. Names of different ranks and circumscriptions have been cited where relevant. Also some synonyms have been

TABLE 1.—Comparison of nomenclature adopted here (left column) with that of other classifications of the genus *Pinus*

Subdivision of genus <i>Pinus</i> L.	Duffield (1952)	Rehder (1949)	Shaw (1914, 1924)	Pilger (1926)
Subgen. 1. <i>Ducampopinus</i> (A. Cheval.) de Ferré Sect. 1. <i>Ducampopinus</i> Subsect. 1. <i>Krempfianae</i> Little & Critchfield			(Group VI)	(Sect. 3, Subsect. 2)
Subgen. 2. <i>Strobus</i> Lemm. Sect. 2. <i>Strobus</i> Subsect. 2. <i>Cembrae</i> Loud. 3. <i>Strobi</i> Loud.	Subgen. <i>Haploxyylon</i>	Subgen. I. <i>Strobus</i> Sect. I. <i>Cembra</i> Ser. 1. <i>Cembrae</i> 2. <i>Flexiles</i> 3. <i>Eustrobi</i>	Sect. A. <i>Haploxyylon</i> Subsect. a. <i>Cembra</i> Group I. <i>Cembrae</i> (II. <i>Flexiles</i>) III. <i>Strobi</i>	Subgen. I. <i>Haploxyylon</i> Sect. 1. <i>Cembra</i> 1. <i>Cembra</i> 2. <i>Strobus</i>
Sect. 3. <i>Parrya</i> Mayr Subsect. 4. <i>Cembroides</i> Engelm. 5. <i>Gerardianae</i> Loud. 6. <i>Balfourianae</i> Engelm.		Sect. II. <i>Parrya</i> Ser. 4. <i>Cembroides</i> 5. <i>Gerardianae</i> 6. <i>Balfourianae</i>	Subsect. b. <i>Paracembra</i> Group IV. <i>Cembroides</i> V. <i>Gerardianae</i> VI. <i>Balfourianae</i>	Sect. 3. <i>Paracembra</i> Subsect. 1. <i>Gerardianae</i> 1. <i>Gerardianae</i> 2. <i>Balfourianae</i>
Subgen. 3. <i>Pinus</i> Sect. 4. <i>Pinea</i> Endl. Subsect. 7. <i>Leiophyllae</i> Loud. 8. <i>Canarienses</i> Loud. 9. <i>Pineae</i> Little & Critchfield	Subgen. <i>Diploxyylon</i>	Subgen. II. <i>Eupitys</i>	Sect. B. <i>Diploxyylon</i> Subsect. c. <i>Parapinaster</i> Group VII. <i>Leiophyllae</i> VIII. <i>Longifoliae</i> IX. <i>Pineae</i>	Subgen. II. <i>Diploxyylon</i> (Sect. 10) Sect. 4. <i>Sula</i> 7. <i>Pinea</i>
Sect. 5. <i>Pinus</i> Subsect. 10. <i>Sylvestres</i> Loud. 11. <i>Australes</i> Loud. 12. <i>Ponderosae</i> Loud. 13. <i>Sabinianae</i> Loud. 14. <i>Contortae</i> Little & Critchfield 15. <i>Oocarpae</i> Little & Critchfield	Subsect. <i>Pinaster</i> Group X. <i>Laricionae</i> Group XI Group XII Group XIII Group XIV	Sect. III. <i>Taeda</i> Ser. 7 <i>Sylvestres</i> 8. <i>Australes</i> 8. <i>Australes</i> 10. <i>Macrocarpae</i> 9. <i>Insignes</i> 9. <i>Insignes</i>	Subsect. d. <i>Pinaster</i> Group X. <i>Laricionae</i> XI. <i>Australes</i> XI. <i>Australes</i> XIII. <i>Macrocarpae</i> XII. <i>Insignes</i> XII. <i>Insignes</i>	Sect. 5. <i>Eupitys</i> 9. <i>Khasia</i> 8. <i>Australes</i> 11. <i>Taeda</i> 10. <i>Pseudostrobus</i> (Sect. 10, 11) 6. <i>Banksia</i> (Sect. 11)

cited under more than one rank. Additional names were proposed in the references cited above. Names in recent classifications published without Latin diagnoses generally have been omitted.

As defined here, the genus *Pinus* contains three subgenera, five sections (three repeating epithets of subgenera), and 15 subsections. Table 1 compares the nomenclature with the classifications by Duffield (1952), Rehder (1949), Shaw (1914, 1924), and Pilger (1926). Duffield and Rehder did not include the entire genus.

Rehder's classification is the same as Shaw's except for slight changes in nomenclature.

Under each subsection are listed in the same order as mapped (Critchfield and Little 1966) the included species with citation, English common name, and brief summary of geographic distribution.

The following key to subdivisions of the genus *Pinus* has been revised and expanded from that of Shaw (1914, p. 25).

KEY TO THE SUBDIVISIONS OF THE GENUS *PINUS*

- A. Leaves narrowly lanceolate, very flattened (1.5-4 mm. wide), 2 in a fascicle; 1 rare species of Vietnam. Subgen. 1. *Ducampopinus* sect. 1. *Ducampopinus* subsect. 1. *Krempfianae*.
- AA. Leaves needlelike, 2-5 (1-8) in a fascicle.
 - B. Bases of fascicle-bracts not decurrent; leaves with 1 vascular bundle, commonly 5 (5-1) in a fascicle, with deciduous sheath; seeds wingless or winged, if long the wing not detachable. Subgen. 2. *Strobilus*.
 - C. Umbo of cone-scale terminal; leaves 5 in a fascicle. Sect. 2. *Strobilus*.
 - D. Cones indehiscent, deciduous at maturity; seeds wingless. Subsect. 2. *Cembrae*.
 - DD. Cones dehiscent at maturity; seeds mostly with long wing or with rudimentary wing. Subsect. 3. *Strobi*.
 - CC. Umbo of cone-scale dorsal; leaves 5-1 in a fascicle. Sect. 3. *Parrya*.
 - E. Seeds large, wingless or with short wing; leaves 4-1 (rarely 5) in a fascicle, spreading.
 - F. Seeds wingless; leaves 4-1 (rarely 5) in a fascicle. Subsect. 4. *Cembroides*.
 - FF. Seeds with short detachable wing; leaves 3 in a fascicle. Subsect. 5. *Gerardianae*.
 - EE. Seeds small, with long wing; leaves 5 in a fascicle, slightly appressed, persistent many years. Subsect. 6. *Balfourianae*.
- BB. Bases of fascicle-bracts decurrent; leaves with 2 vascular bundles, mostly 2 or 3 (sometimes 4-8) in a fascicle, mostly with persistent sheath; seeds mostly with long detachable wing. Subgen. 3. *Pinus*.
- G. Seeds with short or long wing, if with long detachable wing the leaves also with deciduous sheath. Sect. 4. *Pinea*.
- H. Seeds small, with long detachable wing; leaves with deciduous sheath, 3-5 in a fascicle. Subsect. 7. *Leiophyllae*.
- HH. Seeds large, wing otherwise; leaves with persistent sheath.
- I. Seeds with long wing not detachable; leaves 3 in a fascicle, long. Subsect. 8. *Canarienses*.
- II. Seeds large, with short detachable wing; leaves 2 in a fascicle. Subsect. 9. *Pineae*.
- GG. Seeds with long detachable wing; leaves with persistent sheath. Sect. 5. *Pinus*.
- J. Cones symmetrical, mostly opening at maturity, shedding or persistent.
- K. Cones small to large, cone-scales ending in a prickle or slightly protuberant; seeds with thin base of wing.
- L. Spring-shoots mostly with 1 whorl of branches (uninodal).
- M. Leaves mostly 2 in a fascicle; cones not leaving basal scales on twig. Subsect. 10. *Sylvestres*.
- MM. Leaves 2-5 (8) in a fascicle; cones often leaving a few basal scales on twig. Subsect. 12. *Ponderosae*.
- LL. Spring-shoots mostly with 2 or more whorls of branches (multinodal); leaves 2-3 in a fascicle. Subsect. 11. *Australes*.
- KK. Cones large, cone-scales very protuberant and ending in long stout point; seeds large, with thick base of wing; leaves 3 or 5 in a fascicle, long. Subsect. 13. *Sabinianae*.
- JJ. Cones mostly oblique, mostly remaining closed, long persistent.
- N. Leaves 2 in a fascicle, short (2-8 cm. long). Subsect. 14. *Contortae*.
- NN. Leaves mostly 3 (2-5) in a fascicle, more than 8 cm. long. Subsect. 15. *Oocarpae*.

Pinus L.

pine

- Pinus* L., Sp. 1000. 1753; Gen. Pl. Ed. 5, 434. 1754; lectotype species: *Pinus sylvestris* L., Sp. Pl. 1000. 1753.
Pinus Tourn., Elem. Bot. 1: 457, t. 355-356. 1694; Inst. Rei Herbar. 1: 585, t. 355-356. 1700.
Pinus [L., emend.] Mill., Gard. Dict. Abridged. Ed. 4, vol. 3. 1754.
Pinus I. *Pinus* Münchhausen, Hausvat. 5: 215. 1770.
Apinus Neck., Elém. Bot. 3: 269. 1790.
Pinus sect. *Peuce* Griseb., Spicil. Fl. Rumel. 2: 347. 1844.
Cembra Opiz, Seznam Rostl. 27. 1852; nom.
Strobis Opiz, Lotos [Prag] 4: 94. 1854.
Caryopitys Small, Fl. Southeast. U.S. 29, 1326. 1903.
Leucopitys Nieuwl., Amer. Midland Nat. 3: 69. 1913.

Evergreen resinous trees (rarely shrubs) with branches in whorls, 1 a year (uninodal) or 2 or more multinodal). Buds compound, of many scales. Leaves and shoots or 2 forms, primary leaves on long shoots and usually scalelike, secondary leaves needlelike (rarely narrowly lanceolate) on dwarf shoots in fascicles of usually 2-5 (rarely 1 or 6-8), persistent, with sheaths of bud-scales at base. Monoecious. Male cones clustered, of many scales. Cone ovoid, rounded, or cylindric, symmetrical or oblique, maturing in 2 (rarely 3) years, composed of many spirally arranged thick woody cone-scales, each in the axil of a bract, often enlarged or ending in a pickle or spine. Seeds usually 2 at base of cone-scale, usually long-winged or wingless, edible; cotyledons 4-15 (20-23 in *P. maximartinezii*). About 94 or more species of the northern hemisphere (one crossing the equator in Sumatra), mostly of temperate regions and tropical mountains. Trees of the genus *Pinus* are easily recognized by the unique character among conifers, the needlelike leaves on dwarf shoots in fascicles of usually 2-5 with a sheath of bud scales at base.

Subgen. 1. *Pinus* subgen. *Ducampopinus* (A. Cheval.) de Ferré²

- Pinus* subgen. *Ducampopinus* (A. Cheval.) de Ferré ex Critchfield & Little, U.S. Dep. Agr. Misc. Pub. 991: 5. 1966. Holotype species: *Pinus krempfii* Lecomte, Paris Mus. Natl. Hist. Nat. Bull. 27: 191, fig. 1921.
Ducampopinus A. Cheval., Rev. Bot. Appl. d'Agr. Trop. 24: 30. 1944. Holotype species: *Pinus krempfii* Lecomte, l. c.
Pinus sous-genre *Ducampopinus* (A. Cheval.) de Ferré, Paris Acad. Sci. Compt. Rend. 236: 228. 1953; not validly published because reference to basionym lacked original publication with page (ICBN Art. 33).

Leaves with 1 vascular bundle, 2 in a fascicle, narrowly lanceolate, very flattened (1.5-4 mm. wide), finely serrulate or entire, with stomata ventral and rarely also dorsal, with resin-ducts

²Folia fasciculo vasculare 1, 2 in fasciculo, anguste lanceolata, valde complanata (1.5-4 mm. late), minute serrulata vel integra, stomatibus ventralibus atque raro dorsalibus, ductis resiniferis subexternis, et vagina mox decidua. Bases bractearum non decurrentes. Strobilus junior solitarius, sine aculeis. Strobili ovoides, symmetricales, aperti postquam maturi. Squamae carinatae, apophysi crassa pyramidata et umbone dorsali. Semina cum ala longa articulata. Lignum modice durum, leviter resinosum, annulis incrementi, sine tracheidiis radiis, albino albo luteolo, ligno interiore colore salmonis. A subgeneribus aliis differt foliis complanatis atque absentia tracheidiarum radiis.

subexternal, and sheath early deciduous. Bases of fascicle-bracts not decurrent. Conelet solitary, without prickles. Cones ovoid, symmetrical, opening at maturity. Cone-scales keeled, with thick, pyramidal apophysis and dorsal umbo. Seeds with long detachable wing. Wood medium-hard, slightly resinous, with growth rings, without ray-tracheids, sapwood yellowish white, heartwood salmon. This subgenus differs from the other subgenera in the flattened leaves and in the absence of ray-tracheids. One section, one subsection, and one species of Vietnam.

Sect. 1. *Pinus* sect. *Ducampopinus*

- Pinus* subgen. *Ducampopinus* sect. *Ducampopinus*. Holotype species: *Pinus krempfii* Lecomte, l. c.
Pinus sect. *Krempfoides* Gaussen, Lab. Forest Toulouse Trav. tome 2, sect. 1. v. 1, pt. 2: 93. 1960; without Latin diagnosis. With characters of the subgenus. (Characteribus subgeneris.) One species of Vietnam.

Subsect. 1. *Pinus* subsect. *Krempfianae* Little & Critchfield

- Pinus* sect. *Ducampopinus* subsect. *Krempfianae* Little & Critchfield, U.S. Dep. Agr. Misc. Pub. 991: 5. 1966; "*Krempfianae*"; holotype (and only) species: *Pinus krempfii* Lecomte, l. c.
Pinus sect. *Krempfoides* "groupe Asiatique" *Krempfii* Gaussen, l. c. 93. 1960; without Latin diagnosis.
With characters of the subgenus and section. (Characteribus subgeneris et sectionis.) One species of Vietnam: *Pinus krempfii* Lecomte, Paris Mus. Natl. Hist. Bull. 27: 191, fig. 1921.

Subgen. 2. *Pinus* subgen. *Strobis* Lemm., emend.³ soft or white pines

- Pinus* subgen. *Strobis* Lemm., Handb. West-Amer. Cone-bearers. Ed. 3, 20. 1895; emend. Holotype species: *Pinus strobus* L., Sp. Pl. 1001. 1753.
Pinus [group] *Strobis* D. Don, Prodr. Fl. Nepal. 54. 1825.
Pinus [group] *Strobis* Sweet, Hort. Brit. ed. 2, 475. 1830. nom. nud.
Pinus sect. *Strobis* Sweet ex Spach, Hist. Nat. Végét. Phan. 11: 394. 1842.
Pinus sect. *Peuce* Griseb., Spic. Fl. Rumel. 2: 347. 1844.
Pinus subgen. *Peuce* Griseb. ex Endl., Syn. Conif. 138. 1847; pro syn.
Pinus Sect. *Haploxylon* Koehne, Deut. Dendrol. 28, 1893.
Pinus div. *Tenuisquamae* Masters, Linn. Soc. London J. Bot. 35: 569. 1904.
Pinus subgen. *Haploxylon* Rehd. in Bailey, Cult. Evergr. 302. 1923.
Pinus Untergatt. *Haploxylon* Koehne ex Pilger in Engler & Prandl, Nat. Pflanzenfam. Ed. 2, 13: 332. 1926.

³Folia fasciculo vasculari 1, plerumque 5 (5-1) in fasciculo, integra vel serrulata, stomatibus dorsalibus atque centralibus aut tantum ventralibus, ductis resiniferis plerumque externis, et vagina decidua. Bractearum bases non decurrentes. Ramuli vernaes ramulorum verticillo 1 (uninodales). Strobili symmetricales, squamis comparate paucis grandibus, umbone terminali vel dorsali, inermi vel mucronato. Semina aut non alata vel ala rudimentaria separabili vel ala longa articulata. Lignum vulgo lene tantum plus minusve resinosum, annulis incrementi obscuris, comparate pallidum et non grave; radorum tracheidae parietibus laevibus.

Pinus subgen. *Strobos* (Sweet) Rehd., Bibliog. Cult. Trees Shrubs 32. 1949.

Pinus sous-genre *Haplopinus* Campo-Duplan, Lab. Forest Toulouse Trav. tome 2, sect. 1, v. 4, art. 1: 92. 1950; *nom.*

Pinus subgen. *Malacopitys* Ishii, Kochi Univ. Nat. Sci. Rpts. 2: 112, 124. 1952; without Latin diagnosis.

Pinus sous-genre *Cembrapinus* de Ferré, Paris Acad. Sci. Compt. Rend. 236: 228. 1953.

Pinus sous-genre *Paracembrapinus* de Ferré, Paris Acad. Sci. Compt. Rend. 236: 228. 1953.

Leaves with 1 vascular bundle, commonly 5 (5-1) in a fascicle, entire or serrulate, with stomata dorsal and ventral or ventral only, with resin-ducts mostly external, and with deciduous sheath. Bases of fascicle-bracts not decurrent. Spring-shoot with 1 whorl of branches (uninodal). Cones symmetrical, of relatively few large cone-scales. Umbo of cone-scale terminal or dorsal, unarmed or ending in a prickle. Seeds wingless, or with rudimentary detachable wing, or with long wing usually not detachable. Wood mostly soft and only slightly resinous, with annual rings obscure, relatively light-colored and light-weight, the ray-tracheids with smooth walls.

Two sections, five subsections, and 31 or more species of North America and Eurasia. Long known as *Pinus* subgen. *Haploxydon*.

Apparently the oldest valid name for the soft or white pines with rank of subgenus is *Pinus* subgen. *Strobos* Lemm. The original circumscription corresponded to *Pinus* subgen. *Strobos* sect. *Strobos*, as defined here. The name *Strobos* was credited to Pliny by Pfeiffer (Nomencl. Bot. 2: 1303. 1874).

Sect. 2. *Pinus* sect. *Strobos* *

Pinus subgen. *Strobos* sect. *Strobos* Holotype species: *Pinus strobus* L., Sp. Pl. 1001. 1753.

Pinus sect. *Quinquefolius* Duhamel, Traité Arbr. Arbust. France 2: 124. 1755. Lectotype species (selected here): *Pinus strobus* L., Sp. Pl. 1001. 1753.

Pinus [group] *Strobos* D. Don, Prodr. Fl. Nepal. 54. 1825.

Pinus [group] *Strobos* Sweet, Hort. Brit. ed 475, 1830; *nom. nud.*

Pinus [group] *Peuce* Sweet, Hort. Brit. ed 2, 475. 1830; *nom. nud.*

Pinus sect. *Quinae* Loud., Arb. Frut. Brit. 4: 2271. 1838. Lectotype species (selected here): *Pinus strobus* L., Sp. Pl. 1001. 1753.

Pinus sect. *Strobos* Sweet ex Spach, Hist. Nat. Végét. Phan. 11: 394. 1842.

Pinus sect. *Cembra* Spach, Hist. Nat. Végét. Phan. 11: 398. 1842.

Pinus sect. *Peuce* Griseb., Spicil. Fl. Rumel. Byth. 2: 347. 1844.

Pinus Sect. *Haploxydon* Koehne, Deut. Dendrol. 28. 1893.

Pinus subgen. *Haploxydon* sect. *Strobi* Shaw ex Komarov. Fl. URSS. 1: 162. 1934; *seksiia*.

Leaves 5 in a fascicle, entire or serrulate, with epidermis and hypodermis distinct and hypodermis uniform. Conelets with scales not ending in point or bristle. Umbo of cone-scale terminal. Wood

*Folia 5, integra vel serrulata, epidermide et hypodermide distincta atque hypodermide uniformi. Strobilorum juvenium squamae non mucronatae; umbone terminali. Radiorum lignorum cellulae foveis magnis.

ray-cells with large pits. This section corresponds to *Pinus* subsect. *Cembra* of Shaw.

Two subsections, with 19 or more species of mostly northern distribution in North America and Eurasia.

Subsect. 2. *Pinus* subsect. *Cembrae* Loud.

stone pine:

Pinus sect. iii. *Quinae* § xiv. *Cembrae* Loud., Arb. Frut. Brit. 4: 2274. 1838. Holotype species: *Pinus cembra* L., Sp. Pl. 1000. 1753.

Pinus sect. I. *Strobos* § 2 *Cembrae* Engelm., St. Louis Acad. Sci. Trans. 4: 176. 1880.

Pinus Sect. *Haploxydon* Subsect. *Cembra* Parl. ex Koehne, Deut. Dendrol. 30. 1893.

Pinus Sect. *Haploxydon* Subsect. *Cembra* Gruppe *Eucembra* Koehne, Deut. Dendrol. 30, 31. 1893.

Pinus subgen. *Strobos* group *Alpinae* Lemm., Handb. West. Amer. Cone-bearers ed. 3, 23. 1895.

Pinus sect. *Haploxydon* subsect. *Cembra* Shaw, Genus *Pinus* 25, 26. 1914.

Pinus sect. *Haploxydon* subsect. *Cembra* group I. *Cembrae* Shaw, Genus *Pinus* 25, 26. 1914.

Pinus subgen. *Haploxydon* sect. *Cembra* ser. *Cembrae* Rehd., Man. Cult. Trees Shrubs ed. 2, 37. 1940; without Latin diagnosis.

Pinus subgen. *Strobos* sect. *Cembra* ser. *Cembrae* Engelm. ex Rehd., Bibliog. Cult. Trees Shrubs 32. 1949.

Leaves 5 in a fascicle, serrulate or entire. Cones indehiscent, deciduous at maturity. Seeds wingless. (Folia 5, serrulata vel integra. Strobili indehiscentes, maturitate decidui. Semina non alata.) Five species of northern and high altitude distribution, four in Eurasia and one in North America: *Pinus koraiensis*, *pumila*, *sibirica*, *cembra*, *albicaulis*.

Eurasia:

Pinus koraiensis Sieb. & Zucc., Fl. Jap. 2: 28, t. 116, fig. 5-6. 1844; exclud. fig. 1-4; Korean pine. Korea to southeastern Siberia and Japan.

Pinus pumila Regel, Index Sem. Hort. Petrop. 1858: 23. 1859; Japanese stone pine. Northeastern Asia from Siberia to Korea and Japan.

Pinus sibirica Du Tour, Nouv. Dict. Hist. Nat. 18: 18. 1803; Siberian stone pine. Western Russia to Siberia and Mongolia.

Pinus cembra L., Sp. Pl. 1000. 1753; Swiss stone pine. Alps and Carpathian Mountains.

North America:

Pinus albicaulis Engelm., Acad. Sci. St. Louis Trans. 2: 209. 1863; whitebark pine. Northwestern United States and western Canada.

Subsect. 3. *Pinus* subsect. *Strobi* Loud.

white pines

Pinus sect. iii. *Quinae* § xv. *Strobi* Loud., Arb. Frut. Brit. 4: 2280. 1838. Holotype species: *Pinus strobus* L., Sp. Pl. 1001. 1753.

Pinus sect. I. *Strobos* § 1. *Eustrobi* Engelm., St. Louis Acad. Sci. Trans. 4: 175. 1880.

- Pinus* Sect. *Haploxyylon* Subsect. *Cembra* Gruppe *Strobis* Spach ex Koehne, Deut. Dendrol. 30. 1893.
- Pinus* subgen. *Strobis* group *Elongatae* Lemm., Handb. West-Amer. Cone-bearers ed. 3, 20. 1895.
- Pinus* sect. *Haploxyylon* subsect. *Cembra* group II. *Flexiles* Shaw, Genus *Pinus* 25, 28. 1914.
- Pinus* sect. *Haploxyylon* subsect. *Cembra* group III. *Strobi* Shaw, Genus *Pinus* 25, 30. 1914.
- Pinus* subgen. *Haploxyylon* sect. *Strobi* Shaw ex Komarov, Fl. URSS. 1: 162. 1934; sektsiia.
- Pinus* subgen. *Haploxyylon* sect. *Cembra* ser. *Flexiles* Rehd., Man. Cult. Trees Shrubs ed. 2, 37. 1940; without Latin diagnosis.
- Pinus* subgen. *Haploxyylon* sect. *Cembra* ser. *Strobi* Rehd., Man. Cult. Trees Shrubs ed. 2, 38. 1940; without Latin diagnosis.
- Pinus* subgen. *Strobis* sect. *Cembra* ser. *Eustrobi* Engelm. ex Rehd., Bibliog. Cult. Trees Shrubs 33. 1949.
- Pinus* subgen. *Strobis* sect. *Cembra* ser. *Flexiles* Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 33. 1949.
- Leaves 5 in a fascicle, serrulate or entire. Cones dehiscent at maturity. Seeds mostly with long detachable wing or with rudimentary wing. (Folia 5, serrulata vel integra. Strobili maturitate dehiscentes. Semina pro patre maxima ala aut longa separabili aut rudimentaria.)
- This subsection includes *Pinus* group *Flexiles* Shaw. Afterwards, Shaw (Arnold Arboretum J. 5: 226-227. 1924) united *Pinus* group *Flexiles* with group *Strobi* because he considered the rudimentary seed wing of the former not sufficiently distinct to permit retention of a separate group.
- Fourteen species of mostly northern distribution, six in North America and eight in Eurasia: *Pinus strobus*, *monticola*, *lambertiana*, *flexilis*, *strobiformis*, *ayacahuite*, *peuce*, *armandii*, *griffithii*, *dalatensis*, *parviflora*, *morrisonicola*, *jenzeliana*, *wangii*.
- North America:
- Pinus strobus* L., Sp. Pl. 1001. 1753; eastern white pine. Eastern United States and southeastern Canada, also a variety in southern Mexico and Guatemala.
- Pinus monticola* Dougl. ex D. Don in Lamb., Descr. Genus *Pinus*, Ed. 3 (8°), vol. 2, unnumbered p. between p. 144 and p. 145. 1832; western white pine. Far Western United States and adjacent Canada.
- Pinus lambertiana* Dougl., Linn. Soc. London Trans. 15: 500. 1827; sugar pine. Far Western United States and adjacent Baja California.
- Pinus flexilis* James, Exped. Rocky Mts. 2: 27, 35. 1823; limber pine. Western United States and adjacent Canada.
- Pinus strobiformis* Engelm. in Wislitz., Mem. Tour. North Mex. 102. 1848; southwestern white pine. Southwestern United States and northern Mexico.
- Pinus ayacahuite* Ehrenb. in Schlecht., Linnaea 12: 492. 1838; Mexican white pine. Mexico and Central America.
- Eurasia:
- Pinus peuce* Griseb., Spicil. Fl. Rumel. Byth. 2: 349. 1844; Balkan pine. Southeastern Europe.
- Pinus armandii* Franch., Paris Mus. Hist. Nat. Nouv. Arch., Sér. 2, 7: 95-96, t. 12. 1885; "armandi"; Armand pine. China to extreme northeastern India, also Taiwan and Japan.
- Pinus griffithii* McClelland in Griffith, Notul. Pl. Asiat. 4: 17. 1854; Icon. Pl. As. 4, t. 365, excl. fig. 1-3. 1854; blue pine. Eastern Afghanistan through Himalaya Mountains to southwestern China.
- Pinus dalatensis* de Ferré, Toulouse Soc. d'Hist. Nat. Bull. 95: 178, figs. 2, 3. 1960. Southern Vietnam.
- Pinus parviflora* Sieb. & Zucc., Fl. Jap. 2: 27, t. 115. 1844; Japanese white pine. Japan.
- Pinus morrisonicola* Hayata, Gard. Chron., Ser. 3, 43: 194. 1908; Taiwan white pine. Taiwan.
- Pinus jenzeliana* Hand.-Mazz., Oesterr. Bot. Ztschr. 80: 337. 1931. Southern China.
- Pinus wangii* Hu & Cheng, Fan Mem. Inst. Biol. Bull., n. s., 1: 191. 1948. Southeastern Yunnan, China.
- Sect. 3. *Pinus* sect. *Parrya* Mayr**
- Pinus* sect. *Parrya* Mayr, Wald. Nordamer. 241, 427. 1890. Holotype species: *Pinus parryana* Engelm., Amer. J. Sci. Arts, Ser. 2, 34: 332. 1862; (*P. quadrifolia* Parl. ex Sudw.).
- Pinus* sect. *Balfouria* Mayr, Wald. Nordamer. 354, 428. 1890.
- Pinus* Sect. *Haploxyylon* Subsect. *Paracembra* Koehne, Deut. Dendrol. 30. 1893.
- Pinus* div. *Crassisquamae* sect. *Integrifoliae* Masters, Linn. Soc. London J. Bot. 35: 570. 1904.
- Pinus* div. *Crassisquamae* sect. *Serratifoliae* Masters, Linn. Soc. London J. Bot. 35: 570. 1904.
- Pinus* Untergatt. *Haploxyylon* Sect. *Paracembra* Koehne ex Pilger in Engler & Prantl, Natürl. Pflanzenfam. ed. 2, 13: 334. 1926.
- Leaves 5-1 in a fascicle, with epidermis and hypodermis similar. Scales of conelets ending in abrupt point or bristle. Umbo of cone-scale dorsal. Wood ray-cells with small pits. (Folia 5-1, epidermide et hypodermide simili. Strobilorum juvenium squamae mucronatae vel aristatae; umbone dorsali. Radium lignorum cellulae foveolis).
- Three subsections, two North American and one Asian, with 12 species. Rehder (1949) adopted for this section the name accepted here, while Shaw (1914) used *Pinus* subsect. *Paracembra* and Pilger (1926) had sect. *Paracembra*.
- Subsect. 4. *Pinus* subsect. *Cembroides* Engelm.**
- pinyons or nut pines
- Pinus* sect. II. *Pinaster* § 3. *Integrifoliae* subsect. *Cembroides* Engelm., St. Louis Acad. Sci. Trans. 4: 176, 178. 1880; "subsection *Cembroides*" on p. 178. Holotype species: *Pinus cembroides* Zucc., K. Bayer. Akad. Wiss. München, Abhandl. Math.-Phys. 1: 392. 1832; Flora [Jena] 15 (2), Beibl. 93. 1832.
- Pinus* Sect. *Haploxyylon* Subsect. *Paracembra* Gruppe *Parrya* Mayr ex Koehne, Deut. Dendrol. 32. 1893.
- Pinus* subgen. *Pinaster* sect. *Terminales* subsect. *Brachyphyllae* Lemm., Handb. West-Amer. Cone-bearers ed. 3, 25. 1895. Lectotype sp. (selected here): *P. edulis* Engelm. in Wislitz., Mem. Tour. North. Mex. 88. 1848.
- Pinus* subgen. *Pinaster* sect. *Terminales* subsect. *Brachyphyllae* group *Edules* Lemm., Handb. West-Amer. Cone-bearers ed. 3, 26. 1895. Holotype species: *Pinus edulis* Engelm., l. c.
- Pinus* sect. *Edules* Kent in Veitch's Man. Conif. Rev. ed. 308. 1900.
- Pinus* sect. *Haploxyylon* subsect. *Paracembra* group IV. *Cembroides* Shaw, Genus *Pinus* 25, 38. 1914.

- Pinus* subgen. *Haploxyton* sect. *Paracembra* ser. *Cembroides* Rehd., Man. Cult. Trees Shrubs ed. 2, 39. 1940; without Latin diagnosis.
- Pinus* subgen. *Strobis* sect. *Parrya* ser. *Cembroides* (Engelm.) Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 34. 1949.
- Pinus* subgen. *Malacopitys* sect. *Nelsonae* Ishii, Kochi Univ. Nat. Sci. Rpts. 2: 113, 118, 125. 1952; without Latin diagnosis.
- Leaves 5-1 in a fascicle, mostly entire, short (2-9 cm. long). Seeds wingless, large. (Folia 5-1, plerumque integra, brevia (2-9 cm. longa). Semina non alata, grandia.) Eight species of dwarf trees and shrubs of semiarid regions of Southwestern United States and Mexico: *Pinus cembroides*, *edulis*, *quadrifolia*, *monophylla*, *culminicola*, *maximartinezii*, *pinceana*, *nelsonii*.
- Incidentally, the epithet subsection *Cembroides* was applied earlier to fossils as *Pinites* B. Pitys 5. *Cembroides* Endl. (Synopsis. Conif. 285: 1847; "subsec. *Cepibroides*" Pfeiffer, Nomencl. Bot. 1874).
- Pinus cembroides* Zucc., K. Bayer. Akad. Wiss., München, Abhandl. Math.-Phys. 1: 392. 1832; Flora [Jena] 15 (2), Beibl. 93. 1832; Mexican pinyon. Mexico and Southwestern United States.
- Pinus edulis* Engelm. in Wislitz., Mem. Tour. North. Mex. 88. 1848; pinyon. Southwestern United States.
- Pinus quadrifolia* Parl. ex Sudw., U.S. Dep. Agr. Div. Forestry Bull. 14: 17. 1897; Parry pinyon. Southern California and northern Baja California.
- Pinus monophylla* Torr. & Frém. in Frém., Rpt. Explor. Exped. Rocky Mts. 319, t. 4. 1845; "monophyllus"; singleleaf pinyon. Western United States and Baja California, Mexico.
- Pinus culminicola* Andresen & Beaman, Arnold Arboretum J. 42: 438, fig. 2-4. 1961; Potosi pinyon. Cerro Potosi, Nuevo Leon, Mexico.
- Pinus maximartinezii* Rzedowski, Ciencia 23: 17, fig. 1-3, t. 2. 1964. Martinez pinyon. Zacatecas, Mexico.
- Pinus pinceana* Gord., Pinet. 204. 1858; Pince pinyon. Northeastern and eastern Mexico.
- Pinus nelsonii* Shaw, Gard. Chron. Ser. 3, 36: 122, fig. 49. 1904; "nelsoni"; Nelson pinyon. Northeastern Mexico.
- Excluded name: *Pinus* sect. ii. *Ternatae* § xi. *Llaveanae* Loud., Arb. Frut. Brit. 4: 2267. 1838. Holotype (and only) species: *Pinus llaveana* Otto ex Loud., Arb. Frut. Brit. 4: 2267, figs. 2177-2179. 1838; rejected as based on a mixture consisting of two entirely discordant elements (Art. 70). The cone described and figured does not belong to the subsection above. In publishing the name *Pinus llaveana* Schiede (ex Schlecht., Linnaea 12: 488. 1838) later in the same year, Schlechtendal noted Loudon's error. *Pinus llaveana* Schiede is a synonym of *Pinus cembroides* Zucc.
- Subsect. 5. *Pinus* subsect. *Gerardianae* Loud.**
- Pinus* sect. ii. *Ternatae* § viii. *Gerardianae* Loud., Arb. Frut. Brit. 4: 2254. 1838. Holotype species: *Pinus gerardiana* Wall. ex D. Don in Lamb., Descr. Genus *Pinus*. Ed. 3 (8°), vol. 2, unnumbered p. between p. 144 and 145, t. 79. 1832.
- Pinus* sect. II. *Pinaster* § 5. *Halepenses* [subsect.] *Gerardianae* Engelm., St. Louis Acad. Sci. Trans. 4: 176. 1880.
- Pinus* sect. *Haploxyton* subsect. *Paracembra* group V. *Gerardianae* Shaw, Genus *Pinus* 25, 40. 1914.
- Pinus* Untergatt. *Haploxyton* Sekt. *Paracembra* Untersekt. *ardianae* Pilger in Engler & Prantl, Natürl. Pflanzenfam. 2, 13: 332. 1926.
- Pinus* subgen. *Haploxyton* sect. *Paracembra* ser. *Gerardianae* Rehd., Man. Cult. Trees Shrubs ed. 2, 40. 1940; without diagnosis.
- Pinus* subgen. *Strobis* sect. *Parrya* ser. *Gerardianae* (Engelm.) Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 35. 1949.
- Leaves 3 in a fascicle, serrulate. Seeds large, with short, detachable wing. (Folia 3, serrulata. Semina grandia, alata separabili.) Two species in south and east Asia: *Pinus gerardiana*.
- Pinus gerardiana* Wall. ex D. Don in Lamb., Descr. Genus *Pinus* Ed. 3 (8°), vol. 2, unnumbered p. between p. 144 and 145, t. 1832; chilgoza pine. Eastern Afghanistan to northern India.
- Pinus bungeana* Zucc. in Endl., Synopsis. Conif. 166. 1847; bark pine. Northern China.
- Subsect. 6. *Pinus* subsect. *Balfourianae* Engelm.**
foxtail pine
- Pinus* sect. II. *Pinaster* § 3. *Integrifoliae* [subsect.] *Balfourianae* Engelm., St. Louis Acad. Sci. Trans. 4: 1880; "Balfouriana." Holotype species: *Pinus balfouriana* Grev. & Balf. in A. Murr., Bot. Exped. Oreg. [Rpt. No. No. 618, t. 1853.
- Pinus* sect. II. *Pinaster* § 3. *Integrifoliae* Engelm., St. Louis Acad. Sci. Trans. 4: 176. 1880. Lectotype species (selected here): *Pinus balfouriana* Grev. & Balf. in A. Murr., l. c.
- Pinus* sect. *Balfouria* Mayr, Wald. Nordamer. 354, 427. 18
- Pinus* Sekt. *Haploxyton* Subsekt. *Paracembra* Gruppe *Balfouriana* Mayr ex Koehne, Deut. Dendrol. 32. 1893.
- Pinus* subgen. *Pinaster* sect. *Terminales* subsect. *Brachyphyllae* group *Plumosae* Lemm., Handb. West-Amer. Cone-bearing ed. 3, 26. 1895.
- Pinus* sect. *Haploxyton* subsect. *Paracembra* group VI. *Balfourianae* Shaw, Genus *Pinus* 25, 42. 1914.
- Pinus* Untergatt. *Haploxyton* Sekt. *Paracembra* Untersekt. *Balfourianae* Pilger in Engler & Prantl, Natürl. Pflanzenfam. e 2, 13: 332. 1926.
- Pinus* subgen. *Haploxyton* sect. *Paracembra* ser. *Balfourianae* Rehd., Man. Cult. Trees Shrubs ed. 2, 40. 1940; without Latin diagnosis.
- Pinus* subgen. *Strobis* sect. *Parrya* ser. *Balfourianae* (Engelm.) Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 35. 1949.
- Leaves 5 in a fascicle, entire, short (2-4 cm. long), slightly appressed, persistent many years. Seeds with long wing, detachable or not. (Folia 5, integra, brevia (2-4 cm. longa), parum appressa per annos multos persistentia. Semina ala longa, interdum autem nunquam separabili.) Two species at high altitudes in Western United States: *Pinus balfouriana*, *aristata*.
- Pinus balfouriana* Grev. & Balf. in A. Murr., Bot. Exped. Oreg. [Rpt. No. 8] No. 618, t. 1853; foxtail pine. California.
- Pinus aristata* Engelm. in Parry & Engelm., Amer. J. Sci. and Arts, Ser. 2, 34: 331. 1862; bristlecone pine. Western United States.
- Pinus* sect. *Pinaster* § 3. *Integrifoliae* Engelm. has not been adopted, as the epithet was not derived from that of a species and as another name of the same date is established in usage.

Subgen. 3. *Pinus* subgen. *Pinus*⁵

hard pines

- Pinus* subgen. *Pinus*. Lectotype species: *Pinus sylvestris* L., Sp. Pl. 1000. 1753.
- Pinus* I. *Pinus* Muenchh., Hausvater 5: 215. 1770.
- Pinus* [group] *Pinus* Sweet, Hort. Brit. Ed. 2, 475. 1830; *nom. nud.*
- Pinus* [subgen.] B. *Pinus* Endl., Synops. Conif. 137. 1847.
- Pinus* subgen. *Pinus* Endl., ex Parl. in DC., Prod. 16 (2): 364, 378. 1868.
- Pinus* Sect. *Diploxylon* Koehne, Deut. Dendrol. 30. 1893.
- Pinus* subgen. *Pinaster* Lemm., Handb. West-Amer. Cone-bearers Ed. 3, 24. 1895.
- Pinus* div. *Crassiquamae* Masters, Linn. Soc. London J. Bot. 35: 570. 1904.
- Pinus* subgen. *Diploxylon* Rehd. in Bailey, Cult. Evergr. 311. 1923.
- Pinus* Untergatt. *Diploxylon* Koehne ex Pilger in Engler & Prantl, Nat. Pflanzenfam. Ed. 2, 13: 336. 1926.
- Pinus* subgen. *Eupitys* (Spach) Rehd., Bibliog. Cult. Trees 35. 1949.
- Pinus* sous-genre *Diplopinus* Campo-Duplan, Lab. Forest Toulouse Trav. tome 2, sect. 1, v. 4, art. 1: 94. 1950; *nom.*
- Pinus* subgen. *Scleropytis* Ishii, Kochi Univ. Nat. Sci. Rpts. 2: 113, 125. 1952; without Latin diagnosis.
- Pinus* sous-genre *Eupinus* Gaussen, Lab. Forest Toulouse Trav. tome 2, sect. 1, v. 1, pt. 2: 94, 1960; without Latin diagnosis.
- Leaves with 2 vascular bundles, mostly 2 or 3 in a fascicle, sometimes 4-8, serrulate, with stomata dorsal and ventral, with resin-ducts mostly medial and internal but in some species (including the type) external, and mostly with persistent sheath. Bases of fascicle-bracts mostly decurrent. Spring-shoots with 1 whorl of branches (uninodal) or 2 or more (multinodal). Cones symmetrical or oblique, of many cone-scales and complex phyllotaxis. Umbo of cone-scale dorsal. Seeds mostly with long detachable wing. Wood hard, resinous, with annual rings distinct, relatively dark colored and heavy, the ray-tracheids with dentate walls.
- Two sections, nine subsections, and about 62 or more species of wide distribution in the northern hemisphere, mostly of temperate regions and tropical mountains.

Sect. 4. *Pinus* sect. *Pinea* Endl.⁶

- Pinus* B. *Pinus* sectio XI. *Pinea* Endl., Synops. Conif. 182. 1847. Holotype species: *Pinus pinea* L., Sp. Pl. 1000. 1753.
- Pinus* sect. *Ternatae* Loud., Arb. Frut. Brit. 4: 2236. 1838; *pro parte*.

⁵ Folia fasciculis vascularibus 2, plerumque 2 vel 3 in fasciculo, terdum 4-8, serrulata, stomatibus dorsalibus et ventralibus, ductis resiniferis plerumque medialibus et internalibus sed in speciebus aliquis (typo includo) externis, vagina vulgo persistenti. Bractearum bases pro parte maxima decurrentes. Ramuli vernaes ramulorum verticillo 1 (uninodales) vel verticillis 2 vel pluribus (multinodales). Strobili symmetricales vel obliqui, squamis multis, phyllotaxe complexa, et umbone dorsali. Semina plerumque ala longa separabili. Lignum durum, resinosum, annulis incrementi manifestis, comparate fuscum et grave; radiorum tracheidae parietibus dentatis.

⁶ Sectio parva heterogenea *Pino* subsectioni *Parapinasteri* similis cujus species omnes uno characterum sequentium *Pini* subgen. *Sirobi* (*Haploxylon*) habent: folia vagina decidua (subsect. *Leiophyllae*), semina ala brevi separabili (subsect. *Pineae*), vel semina ala longa non articulata (subsect. *Canarienses*). Folia plerumque 3 (2-5). Ramuli vernaes ramulorum verticillo 1 (uninodales). Radiorum ligneorum cellulae foveolis.

Pinus sect. *Sula* Mayr, Wald. Nordamer. 428. 1890.

Pinus sect. *Diploxylon* subsect. *Parapinaster* Shaw, Gen. *Pinus* 25, 44. 1914.

Pinus subgen. *Diploxylon* sect. *Parapinaster* Rehd. in Bailey, Cult. Evergr. 311. 1923.

A small heterogeneous section corresponding to *Pinus* subsect. *Parapinaster* Shaw, all species having one of the following characters of *Pinus* subgen. *Strobis* (*Haploxylon*): Leaves with deciduous sheath (subsect. *Leiophyllae*), seeds with short detachable wing (subsect. *Pineae*), or seeds with long wing not detachable (subsect. *Canarienses*). Leaves mostly 3 (2-5) in a fascicle. Spring-shoots with 1 whorl of branches (uninodal). Wood ray-cells with small pits.

Three subsections and five species of mostly southern distribution in North America and Eurasia.

Pinus canariensis and *P. roxburghii* (as *P. longifolia*) of this section were originally included in the 19 species of *Pinus* sect. *Ternatae* Loud., characterized merely by leaves 3 in a sheath. The remaining 17 species are in *Pinus* subgen. *Pinus* sect. *Pinus*. Accordingly, for *Pinus* sect. *Ternatae* Loud. the lectotype selected here is *Pinus taeda* L. (Sp. Pl. 1000. 1753), the first species cited. Thus, Loudon's name is typified for the larger and more representative group of 3-needle pines and is reduced to synonymy under *Pinus* subgen. *Pinus* sect. *Pinus*. The epithet *Ternatae*, though adopted earlier (Critchfield and Little 1966), would be objectionable for a section. It is not a substantive (Rec. 21B) and not derived from the epithet of a specific name, as are all the other epithets of subdivisions in the genus *Pinus* accepted here.

Subsect. 7. *Pinus* subsect. *Leiophyllae* Loud.

Pinus sect. iii. *Quinae* § xiii. *Leiophyllae* Loud., Arb. Frut. Brit. 2273. 1838. Holotype species: *Pinus leiophylla* Schiede & Deppe in Schlecht. & Cham., Linnaea 6: 354. 1831.

Pinus sect. *Diploxylon* subsect. *Parapinaster* group VII. *Leiophyllae* Shaw, Genus *Pinus* 25, 44. 1914.

Leaves with deciduous sheath, 3-5 in a fascicle. Seeds small, with long detachable wing. (Folia vagina decidua, 3-5. Semina ala longa separabili.) Two species in Mexico, one of these with a variety extending to Southwestern United States: *Pinus leiophylla* (var. *chihuahuana*), *lumholtzii*.

Pinus leiophylla Schiede & Deppe in Schlecht. & Cham., Linnaea 6: 354. 1831; Chihuahua pine. Mexico and Southwestern United States.

Pinus lumholtzii Robins. & Fern., Amer. Acad. Proc. 30: 122. 1895; Lumholtz pine. Mexico.

Subsect. 8. *Pinus* subsect. *Canarienses* Loud.

Pinus sect. ii. *Ternatae* § x. *Canarienses* Loud., Arb. Frut. Brit. 4: 2261. 1838. Holotype species: *Pinus canariensis* C. Smith in Buch, Phys. Besch. Canar. Ins. 159. 1825.

Pinus sect. *Diploxylon* subsect. *Parapinaster* group VIII. *Longifoliae* Shaw, Genus *Pinus* 25, 46. 1914.

Leaves long (20-30 cm.), 3 in a fascicle, with persistent sheath. Seeds large, with long wing not detachable. (Folia longa (20-30 cm.), 3, vagina persistenti. Semina magna, ala longa non articulata.) Designated previously as *Pinus* group *Longifoliae* Shaw. One species in Canary Islands and one in Himalayas: *Pinus canariensis*, *roxburghii*.

Pinus canariensis C. Smith in Buch, Phys. Besch. Canar. Ins. 159. 1825; Canary Island pine. Canary Islands.

Pinus roxburghii Sarg., Silva No. Amer. 11: 9. 1897; chir pine. Himalaya Mountains.

Subsect. 9. *Pinus* subsect. *Pineae* Little & Critchfield

Pinus subgen. *Pinus* sect. *Pineae* Endl. subsect. *Pineae* Little & Critchfield, subsect. nov. Holotypus (and only) species: *Pinus pinea* L., Sp. Pl. 1000. 1753.

Pinus B. *Pinus* sectio XI. *Pineae* Endl., Synops. Conif. 182. 1847.
Pinus l. Gruppe *Pineae* Endl. ex K. Koch, Dendrol. 2 (2): 270. 1873.

Pinus sect. *Pinaster* § 1. *Pineae* Eichler in Engler & Prantl, Natürl. Pflanzenfam. II, 1: 71. 1889; *pro parte*; *nom illegit.* containing type species of genus, *P. sylvestris* L.

Pinus sect. *Diploxylon* subsect. *Parapinaster* group IX. *Pineae* Shaw, Genus *Pinus* 25, 48. 1914.

Pinus Untergatt. *Diploxylon*: Sekt. *Pineae* Endl. ex Pilger in Engler & Prantl, Natürl. Pflanzenfam. Ed. 2, 13: 336. 1926.

Pinus subgen. *Scleropitys* sect. *Pineae* Shaw ex Ishii, Kochi Univ. Nat. Sci. Rpts. 2: 114, 118, 125. 1952; without Latin diagnosis.

Pinus subgen. *Pinus* sect. *Ternatae* subsect. *Pineae* Shaw ex Critchfield & Little, U.S. Dep. Agr. Misc. Pub. 991: 11. 1966; *nomen*.

Leaves 2 in a fascicle, with persistent sheath. Seeds large (15-18 mm.) with short detachable wing. (Folia 2 in fasciculo, vagina persistenti; semina grandia (15-18 mm.), ala brevi separabili.) One species in Mediterranean region: *Pinus pinea* L., Sp. Pl. 1000. 1753; Italian stone pine.

Pinus subsect. *Pineae* is published here as a new subsection because no other name is available for the taxonomic group with this circumscription and rank. Shaw (Genus *Pinus* 24. 1914) credited *Pinus* group *Pineae* to Engelmann (St. Louis Acad. Sci. Trans. 175. 1880), who in a key did not give a name to the group containing this single Mediterranean species. *Pinus* group *Pineae* Shaw was published without clear indication of rank (Art. 4, 35). Ishii (Kochi Univ. Nat. Sci. Rpts. 2: 114, 118, 125. 1952) assigned the rank section but without Latin diagnosis. However, *Pinus* sect. *Pineae* Shaw ex Ishii must be treated as a new name because Shaw's rank "group" was within his rank's section and subsection and was not intended to be a section. Shaw's name could not be assigned to a subsection (Art. 35, Note).

Sect. 5. *Pinus* sect. *Pinus* *

Pinus subgen. *Pinus* sect. *Pinus*. Holotype species: *Pinus sylvestris* L., Sp. Pl. 1000. 1753.

Pinus sect. *Bifoliis* Duhamel, Traité Arbr. Arbust. France 2: 124. 1755. Lectotype species (selected here): *Pinus sylvestris* L., Sp. Pl. 1000. 1753.

Pinus sect. *Trifoliis* Duhamel, Traité Arbr. Arbust. France 2: 124. 1755. Lectotype species (selected here): *Pinus palustris* Mill., Gard. Dict. Ed. 8, *Pinus* No. 14. 1768.

Pinus sect. i. *Binae* Loud., Arb. Frut. Brit. 4: 2152. 1838. Lectotype species (selected here): *Pinus sylvestris* L., Sp. Pl. 1000. 1753.

Pinus sect. i. *Binae* § iii. *Pinaster* Loud., Arb. Frut. Brit. 4: 2213. 1838.

* Folia plerumque 2 vel 3, interdum 4 vel 5 (raro 6-8), vagina persistenti, Ramuli vernaes ramulorum verticillis 2 vel pluribus (multinodales) vel 1 (uninodales). Semina ala longa separabili, maximam partem exilia (crassa atque interdum brevia in subsect. *Sabinianis*). Radiorum ligneorum cellulae plerumque foveolis (foveis magnis in subsect. *Sylvestribus*).

Pinus sect. ii. *Ternatae* Loud., Arb. Frut. Brit. 4: 2235. 1 *pro parte*. Lectotype species (selected here): *Pinus taeda* Sp. Pl. 1000. 1753.

Pinus sect. *Eupitys* Spach, Nat. Sys. Végét. Phan. 11: 374. 1 Lectotype species (selected here): *Pinus sylvestris* L., Sp. 1000. 1753.

Pinus sect. *Taeda* Spach, Hist. Nat. Végét. Phan. 11: 387. 1 *Pinus* sect. *Pinaster* Koch, Syn. Fl. Germ. Helv. Ed. 2, 2: 1844 (not seen).

Pinus sect. *Pinaster* Endl., Synops. Conif. 166. 1847.

Pinus sect. *Pseudo-strobus* Endl. Synops. Conif. 151. 1847.

Pinus sect. *Banksia* Mayr, Wald Nordamer. 107, 426. 1890

Pinus subgen. *Pinaster* sect. *Terminales* Lemm., Handb. W. Amer. Cone-bearers ed. 3, 25. 1895.

Pinus subgen. *Pinaster* sect. *Laterales* Lemm., Handb. W. Amer. Cone-bearers ed. 3, 37. 1895.

Pinus div. *Crassiquamae* sect. *Cubensis* Masters, Linn. S. London J. Bot. 35: 570. 1904.

Pinus div. *Crassiquamae* sect. *Filiifoliae* Masters, Linn. S. London J. Bot. 35: 570. 1904.

Pinus div. *Crassiquamae* sect. *Indicae* Masters, Linn. Soc. London J. Bot. 35: 570. 1904.

Pinus div. *Crassiquamae* sect. *Ponderosae* Masters, Linn. S. London J. Bot. 35: 570. 1904.

Pinus div. *Crassiquamae* sect. *Sylvestres* Masters, Linn. S. London J. Bot. 35: 571. 1904.

Pinus sect. *Jeffreya* Mayr, Fremdl. Wald. Parkbäume 364: 190

Pinus Sekt. *Murraya* Mayr, Fremdl. Wald. Parkbäume 356. 190

Pinus sect. *Diploxylon* subsect. *Pinaster* (Endl.) Shaw, Ge *Pinus* 25, 44. 1914.

Pinus Untergatt. *Diploxylon* Sekt. *Australes* (Loud.) Pilger Engl. & Prantl, Nat. Pflanzenfam. ed. 2, 13: 336. 1926.

Pinus subgen. *Diploxylon* sect. *Laricionis* Shaw ex Komarov, Fl. URSS. 1: 165. 1934.

Pinus subgen. *Diploxylon* sect. *Insignes* Shaw ex Komarov, F URSS. 1: 165. 1934.

A large group corresponding to *Pinus* subsect. *Pinaster* (Endl.) Shaw and containing about three-fifths of the species in the genus. Leaves mostly 2 or 3, sometimes 4 or 5 (rarely 6-8) in a fascicle with persistent sheath. Spring-shoots with 2 or more whorls of branches (multinodal) or 1 (uninodal). Seeds with long detachable wing, mostly thin (thick and sometimes short in subsect. *Sabinianae*). Wood ray-cells mostly with small pits (large in subsect. *Sylvestres*).

Six subsections and 62 or more species, mostly southern in distribution, a few including the type far northern.

Subsect. 10. *Pinus* subsect. *Sylvestres* Loud., emend. *

Pinus sect. i. *Binae* § i. *Sylvestres* Loud., Arb. Frut. Brit. 4: 2152. 1838; emend. Holotype species: *Pinus sylvestris* L., Sp. Pl. 1000. 1753.

Pinus sect. i. *Binae* § ii. *Laricio* Loud., Arb. Frut. Brit. 4: 2200. 1838. Holotype species: *Pinus laricio* Poir., Encycl. Méth. Bot. 4: 399. 1804 (*P. nigra* Arnold).

* Folia pro parte maxima 2, hypodermide uniformi atque ductis resiniferis plerumque externis et medialibus. Ramuli vernaes ramulorum verticillo 1 (uninodales). Strobili plerumque symmetricales, maturitate dehiscentes, decidui vel persistentes. Radiorum ligneorum cellulae foveis magnis. Species subsectionis huius a ceteris chromosomatum heterobrachialium paribus 2 vice 1 different.

- Pinus* sect. i. *Binae* § iii. *Pinaster* Loud., Arb. Frut. Brit. 4: 2213. 1838. Holotype species: *Pinus pinaster* Ait., Hort. Kew. 3: 367. 1789.
- Pinus* sect. i. *Binae* § iv. *Halepenses* Loud., Arb. Frut. Brit. 4: 2231. 1838. Holotype species: *Pinus halepensis* Mill., Gard. Dict. Ed. 8, *Pinus* No. 8. 1768.
- Pinus* sect. *Pinea* A. *Pinaster* (Endl.) Parl. in DC., Prodr. 16 (2): 378. 1868.
- Pinus* sect. II. *Pinaster* § 4. *Sylvestres* Engelm., St. Louis Acad. Sci. Trans. 4: 176. 1880.
- Pinus* sect. II. *Pinaster* § 6. *Pondersosae* [subsect.] *Laricionis* Engelm., St. Louis Acad. Sci. Trans. 4: 177. 1880.
- Pinus* Sect. *Diploxylon* Subsect. *Pinaster* Mayr ex Koehne, Deut. Dendrol. 34. 1893.
- Pinus* sect. *Diploxylon* subsect. *Pinaster* group X. *Laricionis* Shaw, Genus *Pinus* 25, 50. 1914.
- Pinus* subgen. *Diploxylon* sect. *Laricionis* Shaw ex Komarov, Fl. URSS. 1: 165. 1934; sektsiia.
- Pinus* subgen. *Diploxylon* sect. *Pinaster* ser. *Laricionis* Rehd., Man. Cult. Trees Shrubs ed. 2, 40. 1940; without Latin diagnosis.
- Pinus* subgen. *Eupitys* sect. *Taeda* ser. *Sylvestres* (Engelm.) Rehd., Bibliog. Cult. Trees Shrubs 35. 1949.
- Leaves mostly 2 in a fascicle, with uniform hypodermis and resin-ducts mostly external and medial. Spring-shoots with 1 whorl of branches (uninodal). Cones mostly symmetrical, opening at maturity, shedding or persistent. Wood ray-cells with large pits. The species of this subsection differ from all other pines in having two pairs of heterobrachial chromosomes rather than a single pair (Saylor 1961, 1964).
- The largest subsection, corresponding to *Pinus* group *Laricionis* of Shaw. Nineteen or more species, all Old World except *Pinus resinosa* and *P. tropicalis*. Loudon's four subsections of the same date are united here under *Pinus* subsect. *Sylvestres*, the epithet derived from the type species of the genus and thus most appropriate.
- Pinus resinosa* Ait., Hort. Kew. 3: 367. 1789; red pine. Southeastern Canada and Northeastern United States.
- Pinus tropicalis* Morelet, Rev. Hort. Côte d'Or 1: 106. 1851; tropical pine. Western Cuba.
- Pinus nigra* Arnold, Reise Mariazell 8; t. 1785; Austrian pine. Southern Europe, Asia Minor, and local in northwestern Africa.
- Pinus heldreichii* Christ, Naturf. Gesell. Basel Verhandl. 3: 549. 1863; Heldreich pine. Balkan peninsula and southern Italy.
- Pinus mugo* Turra, Gior. Ital. (Grisilini) 1: 152. 1764; Swiss mountain pine. Central and southern Europe.
- Pinus pinaster* Ait., Hort. Kew. 3: 367. 1789; maritime pine. Southwestern Europe and northwestern Africa.
- Pinus halepensis* Mill., Gard. Dict. ed. 8, *Pinus* No. 8. 1768; Aleppo pine. Southern Europe and northern Africa.
- Pinus brutia* Ten., Prodr. Fl. Nap. lxxii. 1811. Southeastern Europe and Asia Minor.
- Pinus sylvestris* L., Sp. Pl. 1000. 1753; Scotch pine. Widespread across Europe and Asia.
- Pinus densiflora* Sieb. & Zucc., Fl. Jap. 2: 22, t. 112. 1844; Japanese red pine. Japan and adjacent eastern Asia.
- Pinus thunbergiana* Franco, Lisboa Inst. Super. Agron. An. 16: 130. 1949; Japanese black pine. Japan and Korea.
- Pinus massoniana* Lamb., Descr. Genus *Pinus* 1: 17, t. 12. 1803; Masson pine. China, northern Vietnam, and Taiwan.
- Pinus taiwanensis* Hayata, Toyko Col. Sci. J. 30 (Art. 1): 307. 1911; Taiwan red pine. Taiwan.
- Pinus luchuensis* Mayr, Bot. Centralbl. 58: 149, fig. 1894; Luchu pine. Ryukyu Islands.
- Pinus hwangshanensis* Hsia in Tsoong, Peiping Nat. Acad. Inst. Bot. Contrib. 4: 155. 1936. Eastern and central China.
- Pinus tabulaeformis* Carr., Traité Gen. Conif. Ed. 2, 510. 1867; Chinese pine. China to southeastern Tibet, Inner Mongolia, Southern Manchuria, and Shantung.
- Pinus yunnanensis* Franch., J. de Bot. 13: 253. 1899; Yunnan pine. Southern China in Yunnan and adjacent provinces.
- Pinus insularis* Endl., Synops. Conif. 157. 1847; Khasi pine. Southeastern Asia and Philippine Islands.
- Pinus merkusii* Jungh. & de Vriese in de Vriese, Pl. Nov. Ind. Bat. 5, t. 2. 1845; Merkus pine. Southeastern Asia, Sumatra, and Philippine Islands.
- Subsect. 11. *Pinus* subsect. *Australes* Loud., emend.***
southern yellow pines
- Pinus* sect. ii. *Ternatae* § ix. *Australes* Loud., Arb. Frut. Brit. 4: 2255. 1838; emend. Holotype species: *Pinus australis* Michx. f., Hist. Arb. Amér. Sept. 1: 64, t. 6. 1810 (*P. palustris* Mill., Gard. Dict. ed. 8, *Pinus* No. 14. 1768).
- Pinus* sect. ii. *Ternatae* § v. *Taeda* Loud., Arb. Frut. Brit. 4: 2236. 1838. Holotype species: *Pinus taeda* L., Sp. Pl. 1000. 1753.
- Pinus* sect. iii. *Quinae* § xii. *Occidentales* Loud., Arb. Frut. Brit. 4: 2271. 1838. Holotype species: *Pinus occidentalis* Sw., Nov. Gen. Sp. Pl. 103. 1788.
- Pinus* sect. *Pinea* B. *Taeda* (Spach) Parl. in DC., Prodr. 16 (2): 390. 1868.
- Pinus* sect. II. *Pinaster* § 8. *Australes* Engelm., St. Louis Acad. Sci. Trans. 4: 177. 1880.
- Pinus* sect. II. *Pinaster* § 7. *Taeda* Engelm., St. Louis Acad. Sci. Trans. 4: 177. 1880.
- Pinus* Sect. *Diploxylon* Subsect. *Taeda* Mayr ex Koehne, Deut. Dendrol. 33: 1893.
- Pinus* sect. *Diploxylon* subsect. *Pinaster* group XI. *Australes* Shaw, Genus *Pinus* 25, 62. 1914.
- Pinus* subgen. *Diploxylon* sect. *Pinaster* ser. *Australes* Rehd., Man. Cult. Trees Shrubs. Ed. 2, 44, 1940; without Latin diagnosis.
- Pinus* subgen. *Eupitys* sect. *Taeda* ser. *Australes* Engelm. ex Rehd., Bibliog. Cult. Trees Shrubs 39. 1949.
- Pinus* subgen. *Diploxylon* subsect. *Pinaster* Group XI Duffield, Ztschr. Forstgefl. Forstpflanz. 1: 95. 1952.
- Leaves 2-3 in a fascicle, with bifiform hypodermis, endodermis with thin outer cell walls, and resin-ducts internal or medial. Spring-shoots mostly with 2 or more whorls of branches (multinodal). Cones symmetrical, opening at maturity, shedding without leaving basal scales on twig; cone-scales with prickles mostly persistent.
- Eleven species, eight in Southeastern and Eastern United States, two in West Indies, and one in both West Indies and adjacent Central America. *Pinus palustris*, *taeda*, *echinata*, *glabra*, *rigida*, *serotina*, *pungens*, *elliottii*, *caribaea*, *occidentalis*, *cubensis*.
- *Folia 2-3, hypodermide biformi, endodermide parietibus externis exilibus, atque ductis resiniferis aut internalibus aut medialibus. Ramuli vernaes plerumque ramulorum verticillis 2 vel pluribus (multinodales). Strobili symmetricales maturitate dehiscetes, decidui sine squamis basalibus in ramulo relictis; squamae mucrone pro parte maxima persistenti.

The southern yellow pines are Group XI of Duffield (1952) and *Pinus* group *Australes* Shaw in part. Loudon's 3 subsections of the same date are united here under *Pinus* subsect. *Australes* to conserve present usage.

Pinus palustris Mill., Gard. Dict. ed. 8, *Pinus* No. 14. 1768; longleaf pine. Southeastern United States.

Pinus taeda L., Sp. Pl. 1000. 1753; loblolly pine. Southeastern United States.

Pinus echinata Mill., Gard. Dict. ed. 8, *Pinus* No. 12. 1768; shortleaf pine. Eastern United States.

Pinus glabra Walt., Fl. Carol. 237. 1788; spruce pine. Southeastern United States.

Pinus rigida Mill., Gard. Dict. ed. 8, *Pinus* No. 10. 1768; pitch pine. Eastern United States.

Pinus serotina Michx., Fl. Bor.-Amer. 2: 205. 1803; pond pine. Southeastern United States.

Pinus pungens Lamb., Ann. Bot. 2: 198. 1805; Table-Mountain pine. Eastern United States.

Pinus elliotii Engelm., Acad. Sci. St. Louis Trans. 4: 186, t. 1-3. 1880; slash pine. Southeastern United States.

Pinus caribaea Morelet, Rev. Hort. Côte d'Or 1: 107. 1851; Caribbean pine. Bahama Islands, Cuba, and Central America.

Pinus occidentalis Sw., Nov. Gen. Sp. Pl. 103. 1788; West Indian pine. Hispaniola and Cuba.

Pinus cubensis Griseb., Amer. Acad. Mem., Ser. 2, 8: 530. 1862; Cuban pine. Cuba.

Subsect. 12. *Pinus* subsect. *Ponderosae* Loud., emend.¹⁰

Pinus sect. ii. *Ternatae* § vi. *Ponderosae* Loud., Arb. Frut. Brit. 4: 2243. 1838; "*Ponderosa*"; emend. Holotype species: *Pinus ponderosa* Laws., Agr. Man. 354. 1836.

Pinus sect. *Pseudo-strobus* Endl., Synops. Conif. 166. 1847.

Pinus sect. *Pinea* C. *Pseudo-strobus* (Endl.) Parl. in DC., Prodr. 16 (2): 398. 1868.

Pinus sect. II. *Pinaster* § 6. *Ponderosae* Engelm., St. Louis Acad. Sci. Trans. 4: 177. 1880.

Pinus Sekt. *Diploxylon* Subsekt. *Pseudostrobus* Mayr ex Koehne, Deut. Dendrol. 33. 1893.

Pinus subgen. *Pinaster* sect. *Terminales* subsect. *Fracticonae* Lemm., Handb. West-Amer. Cone-bearers ed. 3, 31. 1895. Lectotype sp. (selected here): *Pinus ponderosa* Laws., Agr. Man. 354. 1836.

Pinus subgen. *Diploxylon* subsect. *Pinaster* Group XII Duffield, Ztschr. Forstgenet. Forstpflanz. 1: 95. 1952.

Pinus sect. *Jeffreya* Mayr, Fremdl. Wald. Parkbäume 364. 1906. Leaves 2-5 (8) in a fascicle, with hypodermis mostly bifiform or multifiform, endodermis mostly with thick outer cell walls, and resin-ducts mostly medial. Spring-shoots mostly with 1 whorl of branches (uninodal). Cones mostly symmetrical, opening at maturity, when shedding often leaving a few basal scales on twig, cone-scales prickly or protuberant.

Thirteen or more species of Western United States (1 extending into Canada), Mexico, and Central America south to Nicaragua:

¹⁰ Folia 2-5 (8), hypodermide plerumque bififormi vel multififormi, endodermide plerumque parietibus externis crassis, atque ductis resiniferis vulgo medialibus. Ramuli vernaes plerumque ramulorum verticillo 1 (uninodales). Strobili plerumque symmetricales maturitate dehiscentes, saepe paucis squamis basalibus in ramulo relictis; squamae mucronatae vel protuberantes.

Pinus ponderosa, *washoensis*, *jeffreyi*, *engelmannii*, *durangensis*, *cooperi*, *montezumae*, *hartwegii*, *michoacana*, *pseudostrobus*, *lasiana*, *teocote*, *lawsonii*.

This subsection is removed from *Pinus* group *Austri* Shaw and is that group in part. More than half the species *Pinus* group *Austri* of Shaw, all those in Western United S Mexico, and Central America, have been removed and plac this additional, natural group. This subsection corresponds to field's (1952) Group XII except for the exclusion of the subsection.

Pinus ponderosa Laws., Agr. Man. 354. 1836; *ponderosa* Western United States, southwestern Canada and northern Me

Pinus washoensis Mason & Stockwell, Madroño 8: 62. 1 Washoe pine. Local in California and Nevada.

Pinus jeffreyi Grev. & Balf. in A. Murr., Bot. Exped. Oreg. [No. 8] 2, t. 1853; Jeffrey pine. Southwestern Oregon, Calif: Western Nevada, and Northern Baja California.

Pinus engelmannii Carr., Rev. Hort., Sér. 4, 3: 227. 1854; "*er manni*"; Apache pine. Southwestern United States and northv ern Mexico.

Pinus durangensis Martínez, Mex. Inst. Biol. An. 13: fig. 1942; Durango pine. Mexico.

Pinus cooperi C. E. Blanco, Mex. Inst. Biol. An. 20: 185, fig. 1949; Cooper pine. Northwestern Mexico.

Pinus montezumae Lamb., Descr. Genus *Pinus* Ed. 3 (8°) 39, t. 22. 1832; Montezuma pine. Mexico and Guatemala.

Pinus hartwegii Lindl., Bot. Reg. v. 25, Misc. 62. 1839; Hart pine. Mexico, Guatemala, and El Salvador.

Pinus michoacana Martínez, Mex. Inst. Biol. An. 15: 1, fig. 1 1944; Michoacán pine. Central and southern Mexico.

Pinus pseudostrobus Lindl., Bot. Reg. v. 25, Misc. 63. 18 Mexico to Nicaragua.

Pinus douglasiana Martínez, Madroño 7: 4, t. 1. 1943. Doug pine. Western Mexico.

Pinus teocote Schiede & Deppe in Schlecht. & Cham., Linnaea 76. 1830. Mexico and Guatemala.

Pinus lawsonii Roelz ex Gord., Pinet. Sup. 64. 1862; Laws pine. Central and southern Mexico.

Subsect. 13. *Pinus* subsect. *Sabinianae* Loud.¹¹

Pinus sect. ii. *Ternatae* § vii. *Sabinianae* Loud., Arb. Fr Brit. 4: 2246. 1838. Holotype species: *Pinus sabinia* Dougl. ex D. Don in Lamb., Descr. genus *Pinus* ed. 3 (8° vol. 2, unnumbered p. between p. 144 and 145, t. 80. 183. *Pinus* group *Sabinea* K. Koch, Dendrologie 2 (2): 312. 1875 "Gruppe."

Pinus subgen. *Pinaster* sect. *Laterales* group *Graves* Lemm Handb. West-Amer. Cone-bearers ed. 3, 37. 1895.

Pinus sect. *Diploxylon* subsect. *Pinaster* group XII *Macrocarpa* Shaw, Genus *Pinus* 25, 90. 1914.

Pinus subgen. *Diploxylon* sect. *Pinaster* ser. *Macrocarpae* Rehd. Man. Cult. Trees Shrubs ed. 2, 47. 1940; without Lati diagnosis.

¹¹ Folia 3 vel 5, longa (15-33 cm.) crassa, hypodermide crasse multififormi vel bififormi, endodermide parietibus externis nunc exilibus nunc crassis, et ductis resiniferis medialibus. Ramuli vernaes ramulorum verticillo 2 vel pluribus (multinodales) vel 1 (uninodales). Strobili juveni magni squamis mucronatis. Strobili magni, symmetricales vel leviter obliqui maturitate dehiscentes, persistentes; squamae valde protuberantes acumine longo crasso acri atque curvo vel stricto. Semina magna, ala base crassa.

Pinus subgen. *Eupitys* sect. *Taeda* ser. *Macrocarpae* Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 41. 1949.

Leaves 3 or 5 in a fascicle, long (15–33 cm. long), and stout, with thick multiform or uniform hypodermis, endodermis with both thin and thick outer cell walls, and medial resin-ducts. Spring-shoots with 2 or more whorls of branches (multinodal) or 1 (uninodal). Conelets large, with sharp-pointed scales. Cones large, symmetrical or slightly oblique, opening at maturity, persistent; cone-scales very protuberant and ending in long stout, sharp, curved or straight point. Seeds large, with thick base of wing.

Three species of California, one extending into Baja California, Mexico: *Pinus sabiniana*, *coulteri*, *torreyana*. This subsection is the same as *Pinus* group *Macrocarpae* Shaw. However, Duffield (1952) united this group with his Group XII.

Pinus sabiniana Dougl. ex D. Don in Lamb., Descr. Genus *Pinus*. Ed. 3 (8°), vol. 2, unnumbered p. between p. 144 and p. 145, t. 80. 1832; Dougl., Linn. Soc. London Trans. 16: 749. 1833. Digger Pine. California.

Pinus coulteri D. Don, Linn. Soc. London Trans. 17: 440. 1836; Coulter pine. California and northern Baja California.

Pinus torreyana Parry ex Carr., Traité Gen. Conif. 326. 1855; Torrey pine. Local in southern California.

Subsect. 14. *Pinus* subsect. *Contortae* Little & Critchfield¹²

Pinus subgen. *Pinus* sect. *Pinus* subsect. *Contortae* Little & Critchfield, U.S. Dep. Agr. Misc. Pub. 991: 15. 1966. Holotype species: *Pinus contorta* Dougl. ex Loud., Arb. Frut. Brit. 4: 2292, fig. 2210–2211, 1838.

Pinus sect. *Banksia* Mayr, Wald. Nordamer. 107, 426. 1890. Holotype species: *Pinus banksiana* Lamb, Descr. Genus *Pinus* 1: 7, pl. 3. 1803.

Pinus Sect. *Diploxylon* Subsect. *Murraya* Mayr ex Koehne, Deut. Dendrol. 33. 1893. Holotype species: *Pinus murrayana* Grev. & Balf. in A. Murr., Bot. Exped. Ore. [Rpt. No. 8] 2, No. 740, t. 1853. (*Pinus contorta* Dougl. ex Loud. var. *murrayana* (Grev. & Balf. in A. Murr.) Engelm. in S. Wats., Bot. Calif. 2: 126. 1879.)

Pinus subgen. *Pinaster* sect. *Terminales* subsect. *Brachyphyllae* group *Parvicarpeae* Lemmon, Handl. West-Amer. Cone-bearers ed. 3, 25. 1895.

Pinus Sect. *Murraya* Mayr, Fremdl. Wald. Parkbaüme 356. 1906.

Pinus subgen. *Diploxylon* subsect. *Pinaster* Group XIII Duffield, Ztschr. Forstgenet. Forstpflanz. 1: 95. 1952.

Pinus Untergatt. *Pinus* Sectio *Banksianoides* Jählig, Willdenowia 3: 346. 1962; without Latin diagnosis.

Pinus Untergatt. *Pinus* Sectio *Banksianoides* Gruppe *Banksiana* Jählig, Willdenowia 3: 347. 1962; without Latin diagnosis.

Leaves two in a fascicle, short (2–8 cm. long), with bifiform hypodermis and mostly medial resin-ducts. Spring-shoots with two or more whorls of branches (multinodal). Cones small (3–8 cm. long), symmetrical or oblique, usually remaining closed and opening late (serotinous), long persistent, the cone-scales mostly with persistent prickle.

Four species, mostly small trees of the United States, one also in

¹²Folia 2, brevia (2–9 cm. longe), hypodermide biforme, ductis resiniferis maxime ex parte medialis. Ramuli vernaes ramulorum verticillis 2 vel pluribus (multinodales). Strobili parvi (3–8 cm. longe), symmetricales vel obliqui, plerumque clausi vel serotini aperti, longe persistentes, squamae plerumque cum aculeo persistente.

Canada, and one in both Canada and Lower California, Mexico: *Pinus banksiana*, *contorta*, *virginiana*, *clausa*.

This new subsection is Duffield's (1952) Group XIII, composed of four similar species which he removed from *Pinus* group *Insignes* Shaw. *Pinus* sect. *Banksia* Mayr was established for a larger group of 11 species including these four. Mayr's name was afterwards changed by its author to *Pinus* sect. *Murraya* Mayr, an illegitimate name substituted for a slightly larger group of 14 species. In the meantime Koehne published *Pinus* subsect. *Murraya* Mayr with 10 species, also illegitimate, including and citing three groups of Engelm. As apparently no epithet with rank of subsection is available for this small group, we have proposed the new name above. The epithet *Banksia* has not been transferred from rank of section to this subsection because it is not a plural adjective (Rec. 21B) and because of its larger circumscription.

Pinus banksiana Lamb., Descr. Genus *Pinus* 1: 7, pl. 3, 1803; jack pine. Northeastern United States and nearly across Canada.

Pinus contorta Dougl. ex Loud., Arb. Frut. Brit. 4: 2292, fig. 2210–2211. 1838; lodgepole pine. Western North America from Yukon and southeastern Alaska to northern Baja California.

Pinus virginiana Mill., Gard. Dict. ed. 8, *Pinus* No. 9. 1768; Virginia pine. Eastern United States.

Pinus clausa (Chapm.) Vasey ex Sarg., U.S. Census, 10th, 1880, vol. 9 (Rpt. Forests No. Amer.): 199. 1884; sand pine. Florida and southern Alabama.

Subsect. 15. *Pinus* subsect. *Oocarpae* Little & Critchfield¹³

Pinus subgen. *Pinus* subsect. *Oocarpae* Little & Critchfield, U.S. Dep. Agr. Misc. Pub. 991: 19. 1966. Holotype species: *Pinus oocarpa* Schiede in Schlecht., Linnaea 12: 491. 1838.

Pinus subgen. *Pinaster* sect. *Lateralis* group *Serotiniae* Lemm., Handb. West-Amer. Conif. ed. 3, 40. 1895.

Pinus sect. *Diploxylon* subsect. *Pinaster* group XII. *Insignes* Shaw, Genus *Pinus* 25, 76. 1914; pro parte.

Pinus subgen. *Diploxylon* sect. *Insignes* Shaw ex Komarov, Fl. URSS. 1: 165. 1934; sektsiia.

Pinus subgen. *Diploxylon* sect. *Pinaster* ser. *Insignes* Rehd., Man. Cult. Trees Shrubs ed. 2, 45. 1940; without Latin diagnosis.

Pinus subgen. *Eupitys* sect. *Taeda* ser. *Insignes* Shaw ex Rehd., Bibliog. Cult. Trees Shrubs 39. 1949.

Pinus subgen. *Diploxylon* subsect. *Pinaster* Group XIV Duffield, Ztschr. Forstgenet. Forstpflanz. 1: 95. 1952.

Pinus subgen. *Scleropyxis* sect. *Radiatae* Ishii, Kochi Univ. Nat. Sci. Rpts. 2: 114, 121, 125. 1952; without Latin diagnosis.

Leaves mostly 3 (2–5) in a fascicle, with hypodermis mostly bifiform, with resin-ducts mostly medial, sometimes internal or septal. Spring-shoots with 2 or more whorls of branches (multinodal) or 1 (uninodal). Cones mostly oblique, remaining closed, long persistent, cone-scales with prickle or protuberant.

Seven species of Mexico, one of these south to Nicaragua, two others north to California, and one also to both California and Oregon: *Pinus radiata*, *attenuata*, *muricata*, *patula*, *greggii*, *oocarpa*, *pringlei*.

¹³Folia plerumque 3 (2–5), hypodermide plerumque biforme, ductis resiniferis maxime ex parte medialis, interdum internalibus vel septalibus. Ramuli vernaes ramulorum verticillis 2 vel pluribus (multinodales) vel 1 (uninodales). Strobili plerumque obliqui, clausi, longe persistentes, squamae cum aculeo vel protuberantes.

This subsection is Duffield's (1952) Group XIV. It contains the seven remaining species of *Pinus* group *Insignes* Shaw after nine species have been removed, four to compose the essentially new subsection *Contortae*, two to subsect. *Sylvestres*, and three to subsect. *Australes*. The group as originally defined to include those species with closed, or serotinous cones, was shown by Duffield to be somewhat artificial.

Pinus group *Insignes* Shaw was assigned the rank of section by Komarov and rank of series by Rehder. This epithet should not be transferred to the rank of subsection because the original circumscription of the group contained the type species of two earlier subsections (Art. 63): *Pinus* sect. i. *Binae* § iii. *Pinaster* Loud., Arb. Frut. Brit. 4: 2213. 1838; *Pinus* sect. i. *Binae* § iv. *Halepenses* Loud., Arb. Frut. Brit. 4: 2231. 1838.

Pinus sect. *Radiatae* Ishii was published without Latin diagnosis for the group with the same circumscription as *Pinus* group *Insignes* Shaw but with 17 species including 1 addition. Thus, this epithet in an illegitimate name (Art. 36, 63, Rec. 72A) should not be adopted here.

Pinus radiata D. Don, Linn. Soc. London Trans. 17: 442. 1836; Monterey pine. Local in central California and Guadalupe Island, Mexico.

Pinus attenuata Lemm., Mining and Sci. Press 64: 45. 1892; knobcone pine. Southwestern Oregon, California, and northern Baja California.

Pinus muricata D. Don, Linn. Soc. London Trans. 17: 441. 1836; bishop pine. California and northern Baja California.

Pinus patula Schiede & Deppe in Schlecht. & Cham., Linnaea 6: 354. 1831; Mexican weeping pine. Eastern Mexico.

Pinus greggii Engelm. ex Parl. in DC., Prodr. 16 (2): 396. 1868; Gregg pine. Northeastern and eastern Mexico.

Pinus oocarpa Schiede in Schlecht., Linnaea 12: 491. 1838. Mexico to Nicaragua.

Pinus pringlei Shaw in Sarg., Trees and Shrubs 1: 211, t. 100. 1905; Pringle pine. Central and southern Mexico.

DISTRIBUTION OF SPECIES OF *PINUS*

Table 2 summarizes the geographic and taxonomic distribution of the 94 species mapped by Critchfield and Little (1966). The totals may be compared with the briefer compilation of 66 species in Shaw's (1914, p. 24) conservative monograph of a half century ago and with the 103 species recognized by Mirov (1967).

Naturally agreement is lacking on the exact number of species in this large, widely distributed genus. Several species united by Shaw are now generally accepted as distinct, while several very different species have been discovered afterwards. Also, perhaps in time the species concept in *Pinus* may have become slightly narrower. For example, Gaussen (1960) accepted 120 species. Further study of several recently named species in less accessible regions would be desirable for verification of their validity. Careful examination of a widespread species often reveals additional geographic variations worthy of recognition though usually at an infraspecific rank rather than specific.

The top line of table 2 shows total number of species of *Pinus* in major geographic and political regions including larger countries and islands. The 94 species are distributed 59 in the New World or North America in the broad sense (including Central

America and West Indies) and 35 in the Old World. is native in both the Old and New World.

The 59 species of the New World may be grouped follows:

Alaska one, also to Canada and Mexico.

Canada nine, all nine also in United States (including in Mexico and one of those also with a variety in Central.

United States 36, including Western United States 23 ern United States 13.

Western United States and also Canada five (including in Mexico).

Eastern United States and also Canada four (including with a variety in Mexico and Central America).

United States and also Mexico 16 (including one also i America).

Mexico 35, including 15 also in Western United States also in Eastern United States.

Mexico 35, including 10 in Baja California (one on G Island only) and 26 in the rest of Mexico.

Baja California 10 (1 on Guadalupe Island only), all 1 Western United States (including 9 also in California, 1 also in Canada and Alaska, but only one also elsewhere in I

Central America 8, including 6 also in Mexico (1 of t in Eastern United States and Canada) and one also in Wes West Indies four, including one also in Central America.

There are several concentrations of many species of *Pinus* region of western North America including Mexico and America has 44 species in 10 subsections. Mexico has 35 spe according to Martínez, 1945), more than any other area (size, and about as many as the United States, though cor only one-fifth of the area of contiguous or conterminous States.

California has 19 of the 23 species of *Pinus* in Western States, probably the greatest concentration in the genus. N. Baja California perhaps should be included, as nine of the 19 southward beyond the artificial, political boundary. Three (*P. balfouriana*, *P. sabiniana*, and *P. torreyana*) are ende California. Eight others have most of their range within Cali Four of these (*P. quadrifolia*, *P. coulteri*, *P. radiata*, *P. mu* are restricted to California and northern Baja California (*P. i* on Guadalupe Island only), while one (*P. attenuata*) exten to western Oregon and two others (*P. lambertiana*, *P. je* also to western Oregon and western Nevada. Another *wasoensis*) is local in California and western Nevada.

The 35 Old World species of *Pinus* include 34 in Eurasia also in northern Africa) and one confined to Canary Islar Africa. The 34 of Eurasia may be grouped into western Euro and Asia 27 (including three also in Western Europe). Afric four species (including three also in Eurasia and one confin Canary Islands). Mainland China has 14 species, Soviet Unio and India 5.

In eastern and southeastern Asia from China and Japan s ward, including islands, are found 21 of the 27 species of i in Asia. Mainland China has 14. Southeastern Asia (excl islands) has five including three not also in China. Korea has Japan six, Taiwan four, Philippines two, and Sumatra one. Asiatic species are limited to islands from Japan to Taiwan not known from the continent.

TABLE 2.—Geographic and taxonomic distribution of the 94 species of *Pinus*, based on Critchfield and Little (1966). Totals in subgenera and sections are in parentheses. The top line summarizes totals in major geographic and political regions, while vertical columns show distribution by taxonomic subdivisions. Many species are in more than 1 column. Species under islands are counted also under adjacent continent except column of southeast Asia

Taxonomic group	World	New World	Canada	United States	Western United States	Eastern United States	Mexico	Mexico (excluding Baja California)	Baja California	Central America	West Indies	Old World	Canary Islands	Africa	Eurasia	Western Europe	U.S.S.R.	Mediterranean Region	Asia	Asia Minor	India	China (mainland)	Japan	Southeast Asia (excluding islands)	Taiwan	Philippines	Sumatra
<i>Pinus</i>	94	59	9	36	23	13	35	26	10	8	4	35	1	4	34	10	6	5	27	5	5	14	6	5	4	2	1
Subgen. 1. <i>Ducampopinus</i>	(1)	(1)										(1)	(1)		(1)	(1)			(1)				(1)	(1)			
Sect. 1. <i>Ducampopinus</i>	(1)											(1)	(1)		(1)	(1)			(1)				(1)	(1)			
Subsect. 1. <i>Kremplianae</i>	1											1			1				1				1				
Subgen. 2. <i>Strobus</i>	(31)	(17)	(4)	(12)	(11)	(1)	(12)	(9)	(4)	(2)		(14)			(14)	(2)	(3)	(12)	(10)		(3)	(7)	(4)	(1)	(2)		
Sect. 2. <i>Strobus</i>	(19)	(7)	(4)	(6)	(5)	(1)	(4)	(3)	(4)	(2)		(12)			(12)	(2)	(3)	(10)	(12)		(2)	(6)	(4)	(1)	(2)		
Subsect. 2. <i>Cembrae</i>	5	1	1	1	1							4			4	1	3	3	3				2	2			
3. <i>Strobi</i>	14	6	3	5	4	1	4	3	1	2		8			8	1		7	7		2	4	2	1	2		
Sect. 3. <i>Parrya</i>	(12)	(10)		(6)	(6)		(8)	(6)	(3)			(2)			(2)			(2)			(1)	(1)					
Subsect. 4. <i>Cembroides</i>	8	8	4	4	4		8	6	3			2			2												
5. <i>Gerardianae</i>	2																										
6. <i>Balfourianae</i>	2	2		2	2																						
Subgen. 3. <i>Pinus</i>	(62)	(42)	(5)	(24)	(12)	(12)	(23)	(17)	(6)	(6)	(4)	(20)	(1)	(4)	(19)	(8)	(3)	(5)	(14)	(5)	(2)	(7)	(2)	(3)	(2)	(1)	
Sect. 4. <i>Pinus</i>	(5)	(2)		(1)	(1)		(2)	(2)				(3)	(1)	(1)	(2)	(1)		(1)	(1)	(1)							
Subsect. 7. <i>Leptophyllae</i>	2	2		1	1		2																				
8. <i>Canarienses</i>	2																										
9. <i>Pinus</i>	1												2	1	1	1	1	1	1	1							
Sect. 5. <i>Pinus</i>	(57)	(40)	(5)	(23)	(11)	(12)	(21)	(15)	(6)	(6)	(4)	(17)		(3)	(17)	(7)	(3)	(4)	(13)	(4)	(1)	(7)	(2)	(3)	(2)	(1)	
Subsect. 10. <i>Sylvestres</i>	19	2	1	1	1	1						1		3	17	7	3	4	13	4	1	7	2	3	2	1	
11. <i>Austrolo</i>	11	11	1	8	4	8																					
12. <i>Ponderosa</i>	13	13	1	4	4	4	12	11	1	4																	
13. <i>Sabinianae</i>	3	3		3	3																						
14. <i>Contorta</i>	4	4	2	4	1	3	1	1	1																		
15. <i>Oocarpae</i>	7	7		3	3		7	4	3	1																	

MAPS OF GEOGRAPHIC DISTRIBUTION OF SUBDIVISIONS

The geographic distribution of the genus *Pinus* and its subdivisions is presented graphically and summarized on 22 maps, eight of the world (maps 1-8) and 14 of North America (maps 9-22). These maps listed under Contents and in the List of Maps have been combined without revision from our 63 maps of the 94 species (Critchfield and Little 1966). Included are maps of the genus, each subgenus, and each subsection, both in the world and North America. However, the eight subsections confined to North America are shown only on North American maps. The five sections have not been mapped separately.

The maps were copied by pencil directly from 63 original maps. A projector in a dark room was used to copy and combine the species maps on the two smaller base maps of North America and the world. Then the maps of the subsections were further combined by tracing on a light table. These pencil maps have been reproduced here as halftones made with a very fine screen. Thus, time-consuming redrafting, with possible loss of detail and accuracy, was avoided.

Map 1 outlines the detailed natural distribution of the genus *Pinus* throughout the world and summarizes the maximum ranges of all 94 species combined. The earlier map of the genus *Pinus* (Critchfield and Little 1966, map 1), had a different projection, viewed from the North Pole.

The genus *Pinus* (maps 1 and 9) is widespread across North America and Eurasia and extends to northern Africa and to adjacent islands of these continents. It is mainly north temperate but crosses the Tropic of Cancer into the tropics southward mostly in mountains in Mexico, Central America, West Indies, and southeastern Asia to the Philippines and Sumatra. One species (*Pinus merkusii*) crosses the Equator to 2° S. latitude. Three species cross the Arctic Circle in Eurasia, and two of these extend beyond 70° N. latitude.

Map 9 shows the natural distribution of the genus *Pinus* in North America, or the New World, and summarizes the maximum ranges of all 59 species. *Pinus* is the most widespread genus of trees in North America. It extends from Newfoundland across Canada to southeastern Alaska, south in Western United States through mountains of Mexico and Central America to Nicaragua (three species), also to Baja California and Guadalupe Island, and through Eastern United States to the Bahamas, Cuba, and Hispaniola. Its latitudinal range is more than 53° from 65° N. in northwestern Canada to 12° N. in Nicaragua. The large area across Canada is occupied mostly by two species, *P. banksiana* and in the Far West and southeastern Alaska *P. contorta*.

The genus *Pinus* is native in 48 States of the United States, all on the continent, including Alaska, with the exception of Kansas. It is planted also but not native in Hawaii and the Commonwealth of Puerto Rico. *P. echinata* was reported many years ago from Kansas (Britton and Shafer, No. Amer. Trees 32, 1908), but the late Frank C. Gates did not find it in a careful search.¹⁴

Mention should be made of the first map of the genus *Pinus* in North America, published 85 years earlier by Sargent (1884). That map indicated approximate limits of the genus, as well as numbers of species. The southeastern corner of Kansas was shown within the range.

¹⁴ Correspondence with senior author.

The distribution of the two main subgenera of *Pinus* (maps 2, 3, 10, 11) has been mapped and summarized by Florin (1963, pp. 252-256). These new maps show in greater detail the limits and the discontinuous portions observed by him. For example, *Pinus* is absent from interior Alaska, interior United States, northeastern Canada, and large areas of central Eurasia. As noted by Florin, the range of *Pinus* subgen. *Strobos* is less than that of subgen. *Pinus* except in northern and northeastern Asia. *Pinus* subgen. *Ducampopinus* is represented by one anomalous species (*Pinus krempfii*) confined to Vietnam (map 2).

The hard pines comprising *Pinus* subgen. *Pinus* reach the extreme limits of the genus except in northern and northeastern Asia. They extend farthest south, to Nicaragua in the New World, to northern Africa, and below the equator to Sumatra. Also, hard pines are the only pines in islands along the southern border of the generic range, for example, in the West Indies, Canary Islands, Sumatra, and Philippines. The presence of pines on these oceanic islands is evidence of migration across water and suggests that some distribution on land may also have been discontinuous. The soft pines forming *Pinus* subgen. *Strobos* occupy mostly cool regions, especially northern latitudes, but extend southward in mountains, including the Cordilleras of Mexico and Central America, the Alps, and Himalayas.

In North America the 42 species of hard pines forming *Pinus* subgen. *Pinus* are more extensive than the 17 species of soft pines composing *Pinus* subgen. *Strobos* (maps 10, 11). The former, occupying the northern and southern limits, are the only pines in the West Indies and are represented in all States except Kansas, Iowa, and Hawaii. The soft pines have a single species (*Pinus strobus*) in Eastern North America but are native in all States except nine: Alaska, Kansas, Missouri, Arkansas, Louisiana, Mississippi, Alabama, Florida, and Hawaii.

The maps of the 15 subsections (maps 4-8, 12-22) show mostly compact or continuous ranges, perhaps more natural than ranges of corresponding groups of older classifications. Eight subsections are confined to North America and four to the Old World, while only three are found in both.

The three subsections represented in both New and Old World are of special interest. *Pinus* subsect. *Cembrae*, with indehiscent cone regarded as primitive, has circumboreal distribution in cold temperate regions (maps 4, 12). Its five species include one at high altitudes in northwestern North America, one in the Alps and Carpathian Mountains, two in northern Asia, and one from Korea and eastern Manchuria to Japan.

Pinus subsect. *Strobi*, the most closely related subsection, is also in both New and Old World but extends southward in less cold regions, especially mountains (maps 5, 13). It contains 14 species, six New World and eight Old World, almost half of the 31 species of the subgenus of soft pines. The New World species extend farthest north, to southern Canada, and south mostly in mountains to Mexico, Central America, and Appalachian Mountains. Old World species are scattered mostly in mountainous parts of southeastern Europe, the Himalayas, mainland China, Vietnam, Taiwan, and Japan.

Pinus subsect. *Sylvestres*, the largest subsection in number of species, is the only one of nine subsections of hard pines found in both the New and Old World (maps 8, 17). However, only two of the 19 species are found in the New World. This large subsection

contains 17 of the 20 hard pine species and almost half of the 35 pine species in the Old World. The species are widespread from northern Africa across Eurasia to Japan. The two New World species are both eastern, one northeastern, and one in Cuba. Cytological studies by Saylor (1961, 1964) confirmed that this is a natural group distinguished from all other species of the genus by the two pairs of heterobrachial chromosomes rather than one pair.

The four subsections confined to the Old World have a total of only six species. *Pinus* subsect. *Gerardianae* contains one species in the Himalayas and nearby mountains and one rare and scattered in mountains of northern China, separated by 1,300 miles of Tibetan highland (map 6). *Pinus* subsect. *Canarienses* has one species in the Canary Islands, off the coast of northwestern Africa, and one in the Himalaya Mountains more than 5,000 miles away (map 7).

Eight subsections with a total of 50 species are limited to the New World. The two subsections of soft pines occupy distinct altitudinal zones. *Pinus* subsect. *Balfourianae* has two timberline species scattered at high altitudes in the Western United States (map 15). *Pinus* subsect. *Cembroides* comprises the pinyons or nut pines, eight species of dwarf trees and shrubs of semiarid regions of low altitudes in Southwestern United States and Mexico, one of these up to timberline (map 14).

One of the five New World subsections of hard pines is eastern. *Pinus* subsect. *Australes* as remodeled is a natural group of 11 species, eight in the Eastern and Southeastern United States, including the southern yellow pines, two in West Indies, and one in both West Indies and Central America (map 18).

Pinus subsect. *Ponderosae* with 13 species is the largest New World subsection (map 19). It extends from southwestern Canada through the Western United States (mostly one species, *Pinus ponderosa*) and Mexico south to Nicaragua. Also, most of the hard pines of Mexico belong here.

Pinus subsect. *Leiophyllae* has two Mexican species, one also in Southwestern United States (map 16). *Pinus* subsect. *Oocarpae* has seven species mostly Mexican, but it extends north along the Pacific coast to California and Oregon and south to Nicaragua (map 22).

Pinus subsect. *Sabinianae* has the smallest range of any New World subsection (map 20). It has three species in California, one extending into Baja California, Mexico.

Pinus subsect. *Contortae* is composed of four species of mostly small trees of mostly separate range around North America, one in Florida, one in Eastern United States, one nearly across Canada, and one western south to Baja California.

APPLICATIONS OF THE MAPS

These maps of subdivisions of the genus *Pinus* may have further applications as suggested, for example, in researches on classification, present and past distribution, tree breeding, and introduction.

There is a relatively close correlation between the latest classification of the genus and the geographic distribution of the subdivisions of the genus *Pinus*. In general the closely related species are grouped in nearby regions. Further improvements in the classification may lead to refinement in the maps of the subdivisions.

To the extent that the classification may be artificial, the maps will be affected.

These maps confirm several familiar patterns of plant distribution and evolution. The genus *Pinus* is north temperate with minor range extensions of some subdivisions southward into the tropics, mostly on mountains. It apparently originated in the north temperate zone. Subsections of broad range may be older than those of local range. The ranges of some subsections now discontinuous may have been continuous in the past, or more nearly so. These subsections of interrupted distribution may be relatively old. However, the common occurrence on islands confirms that some discontinuous range may be normal.

The three subsections with representatives in both the Old and New World apparently are older and more primitive than most of the remaining 12 sections.

Six subsections with 35 species are restricted to the region of Western North America including Mexico and Central America. It may be assumed that these six subsections and their species evolved here, in the absence of fossil evidence to the contrary. Speciation in this region may have been accelerated by the highly variable and changing conditions of altitude, climate, and soil. The subsections regarded as more specialized or advanced generally occupy small areas within this region and may be relatively young in origin. Examples are *Pinus* subsect. *Cembroides*, subsect. *Sabinianae*, and subsect. *Oocarpae*.

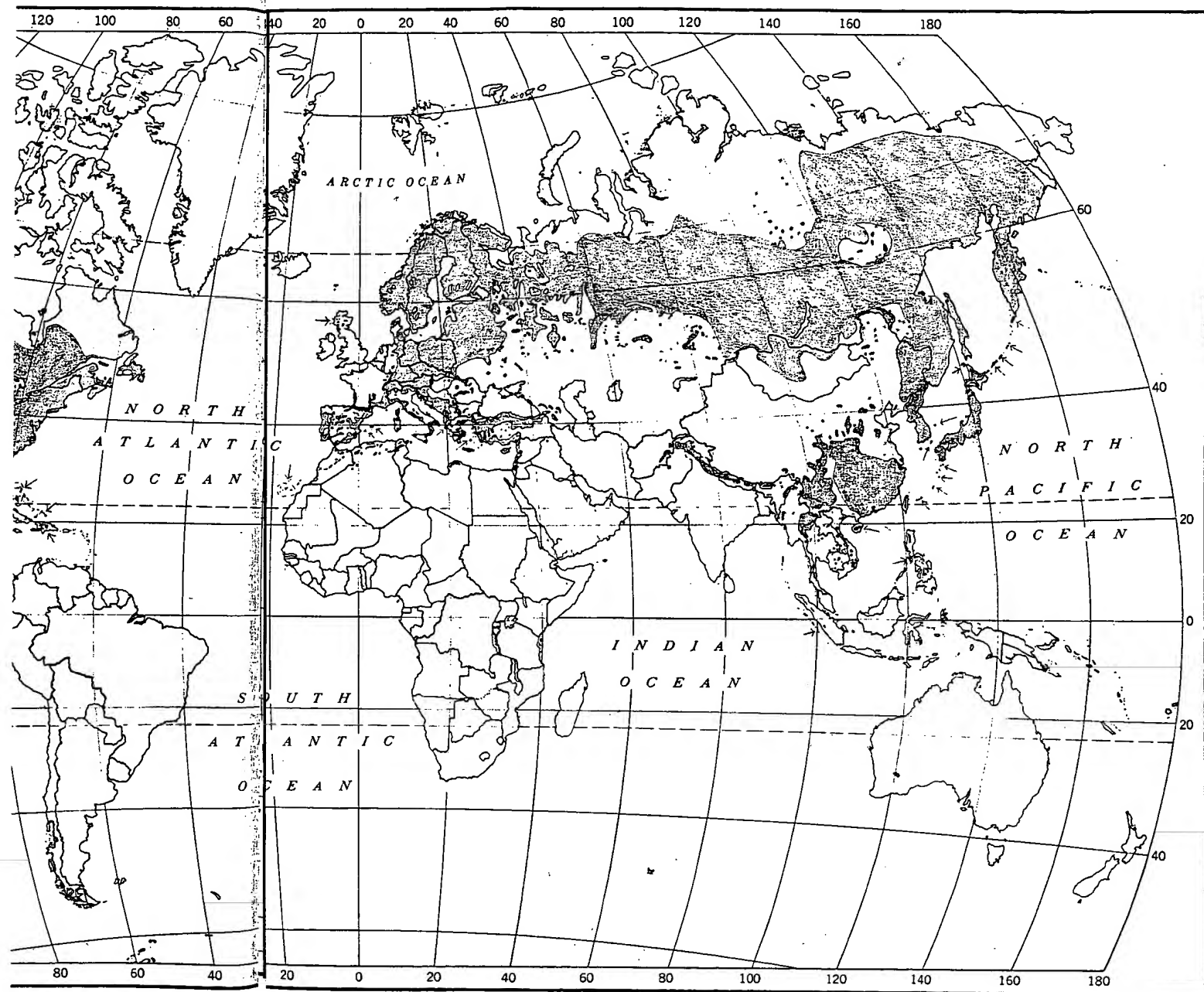
Florin (1963, pp. 252-256) has reviewed and mapped the present and past distribution of the subgenera of the genus *Pinus*. However, he did not consider subdivisions of lower rank because of the difficulty of classifying fossil remains correspondingly. As more information about fossil pines becomes available, perhaps further comparison can be made with the maps of subsections in studies of evolution within the genus. Fossil records should contribute to better interpretation of the present maps, such as in connecting discontinuous parts and in confirming routes and periods of migration.

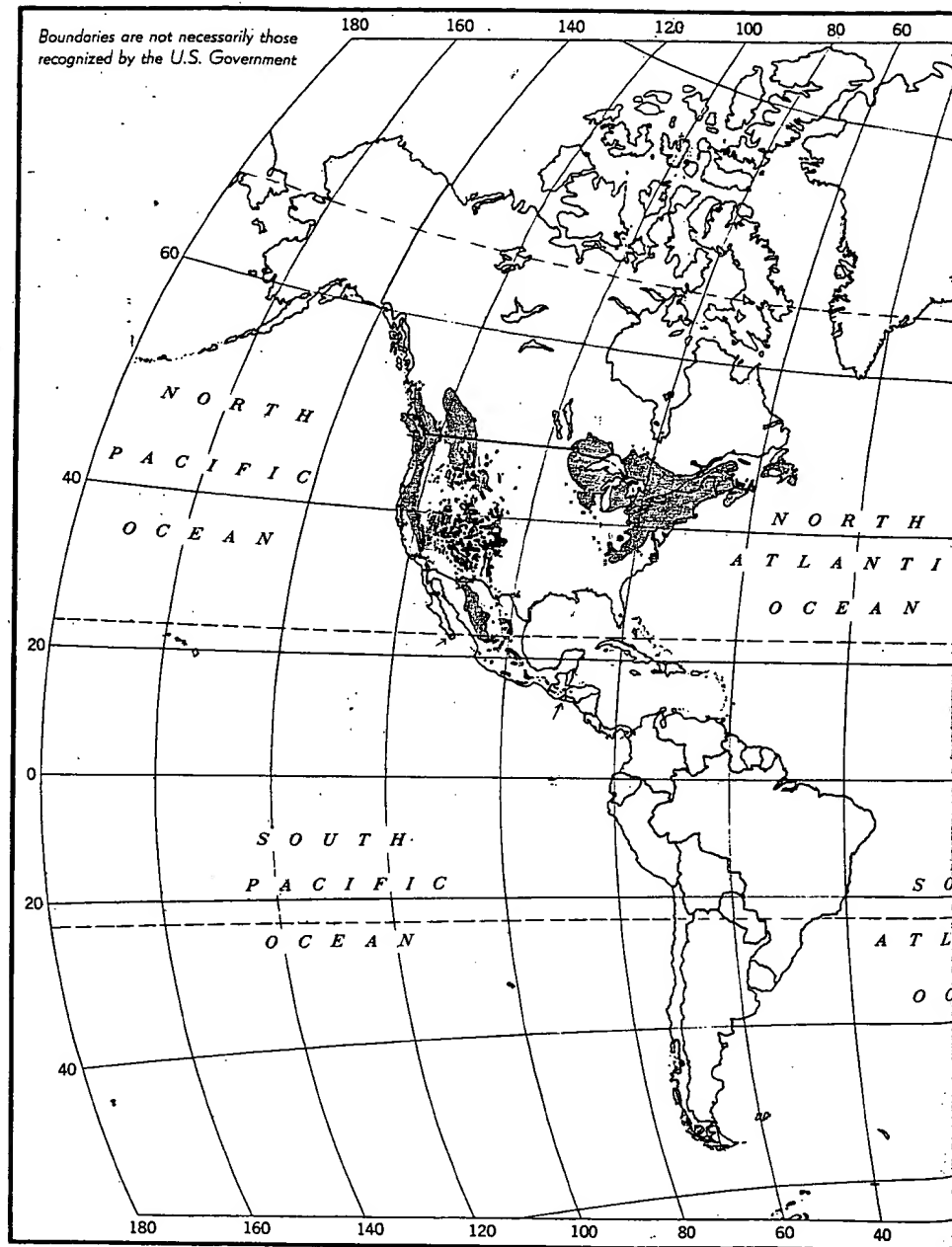
Florin noted that large scale fluctuations in distribution have occurred as a consequence of changes in the relation of land and sea, in topography, and in climatic conditions since early Cretaceous times, the age of the oldest known fossil remains of *Pinus* subgen. *Pinus*. Very few fossil records are outside present areas of distribution, but *Pinus* subgen. *Strobus* was more widespread in Europe in the Tertiary than now. Florin stated that the extension of *Pinus* subgen. *Pinus* into Central America probably dates from the middle to late Tertiary, and its present configuration in the West Indian, Mediterranean, and Malaysian regions from Pleistocene times.

The maps of subsections may suggest possible areas of geographic races or provenances in selecting pines for introduction to other parts of the world with similar climates. As related species of the same subsection have similar properties, additional geographic sources may be indicated.

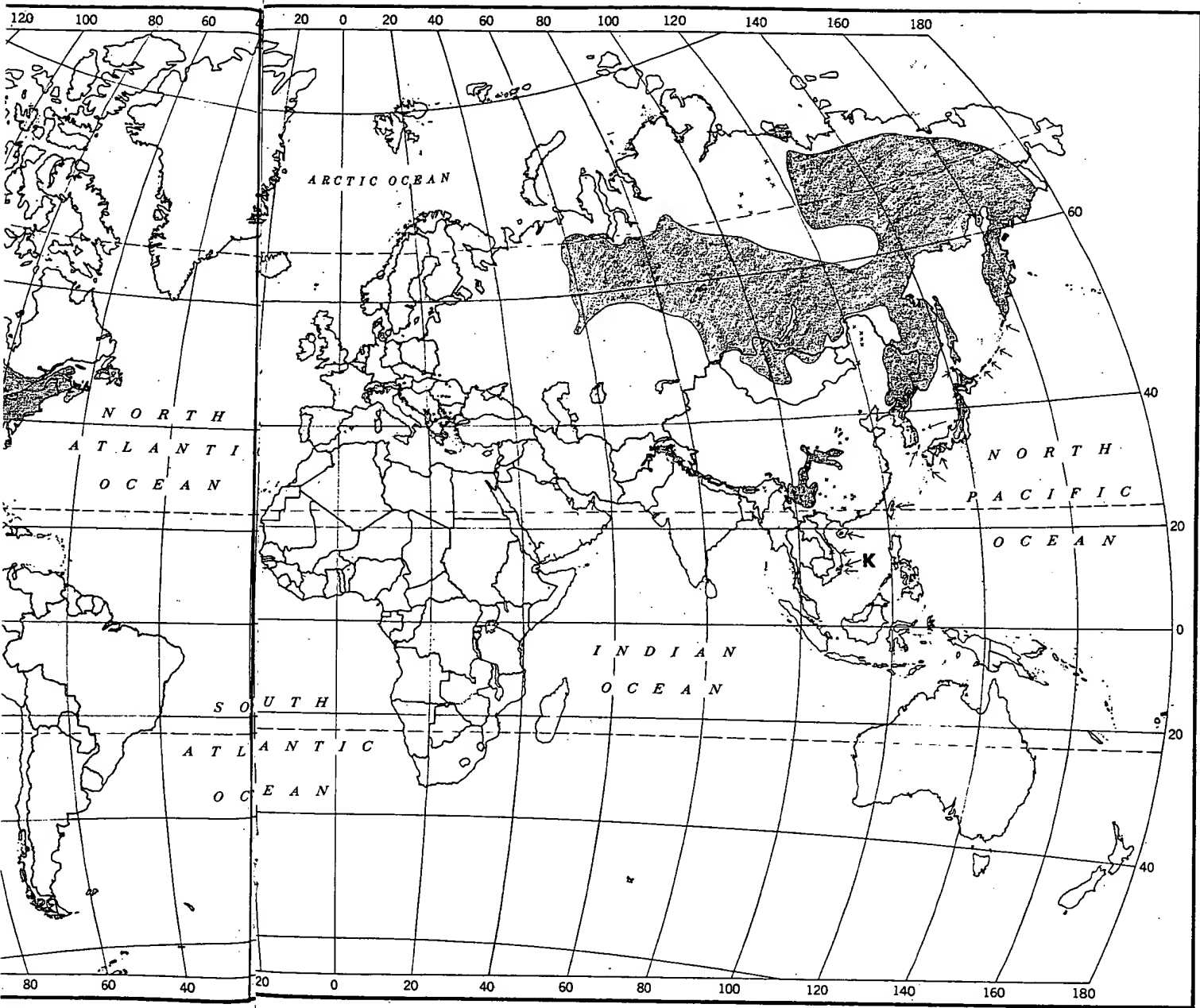
There may be applications in tree breeding programs also. In general, artificial hybridization is likely to be more successful between closely related species of the same subsection than between those of different subsections. Each map of a subsection summarizes the geographic distribution of related species within which crossing might be possible. Thus, the maps may suggest localities as sources of germ plasm for hybridization for regions with similar climates elsewhere.







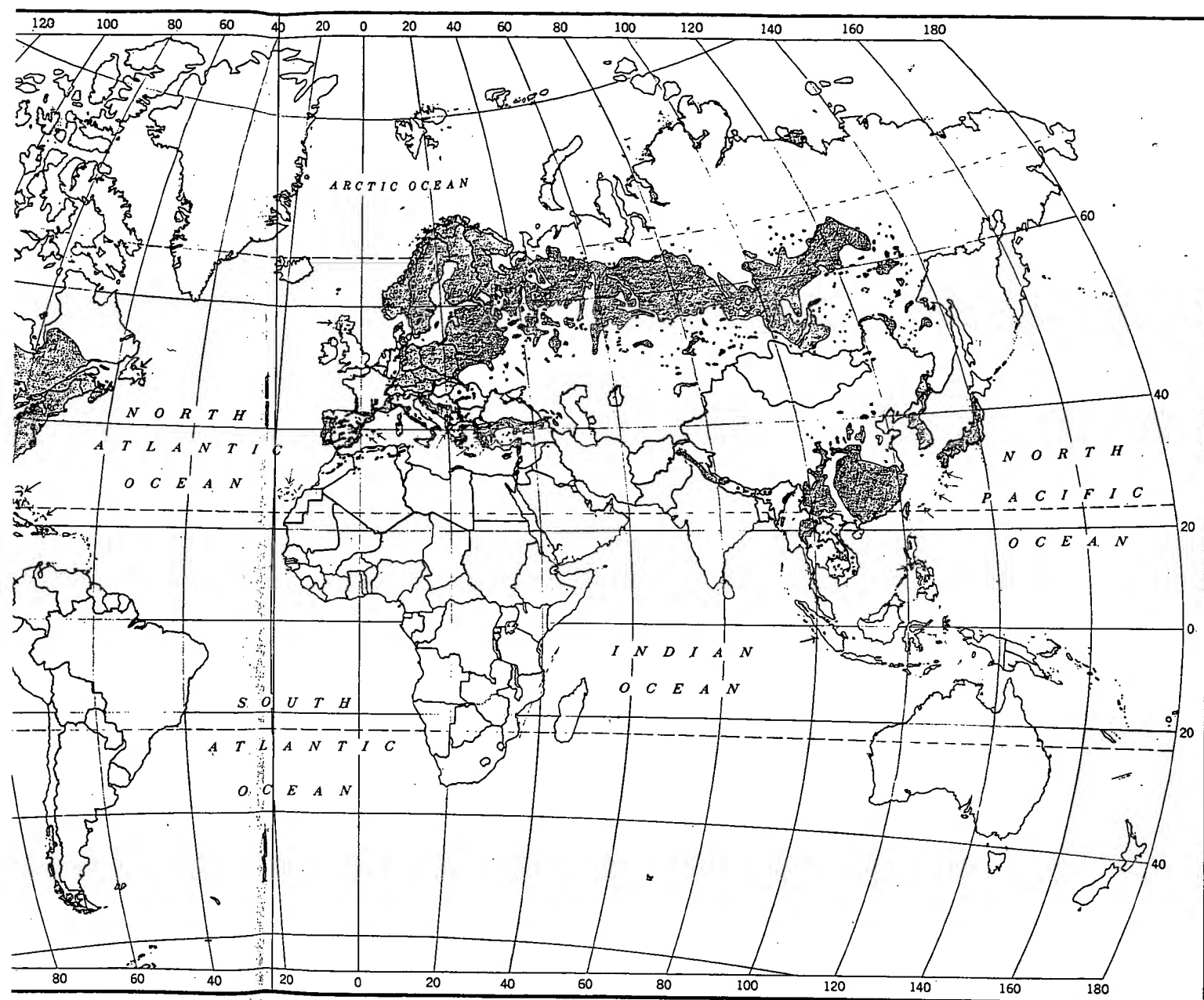
Map 2. *Pinus* subgen. *Strobos* (31 species) and subgen. *Ducampopinus* (1 species, *P. krempfii*, K.).

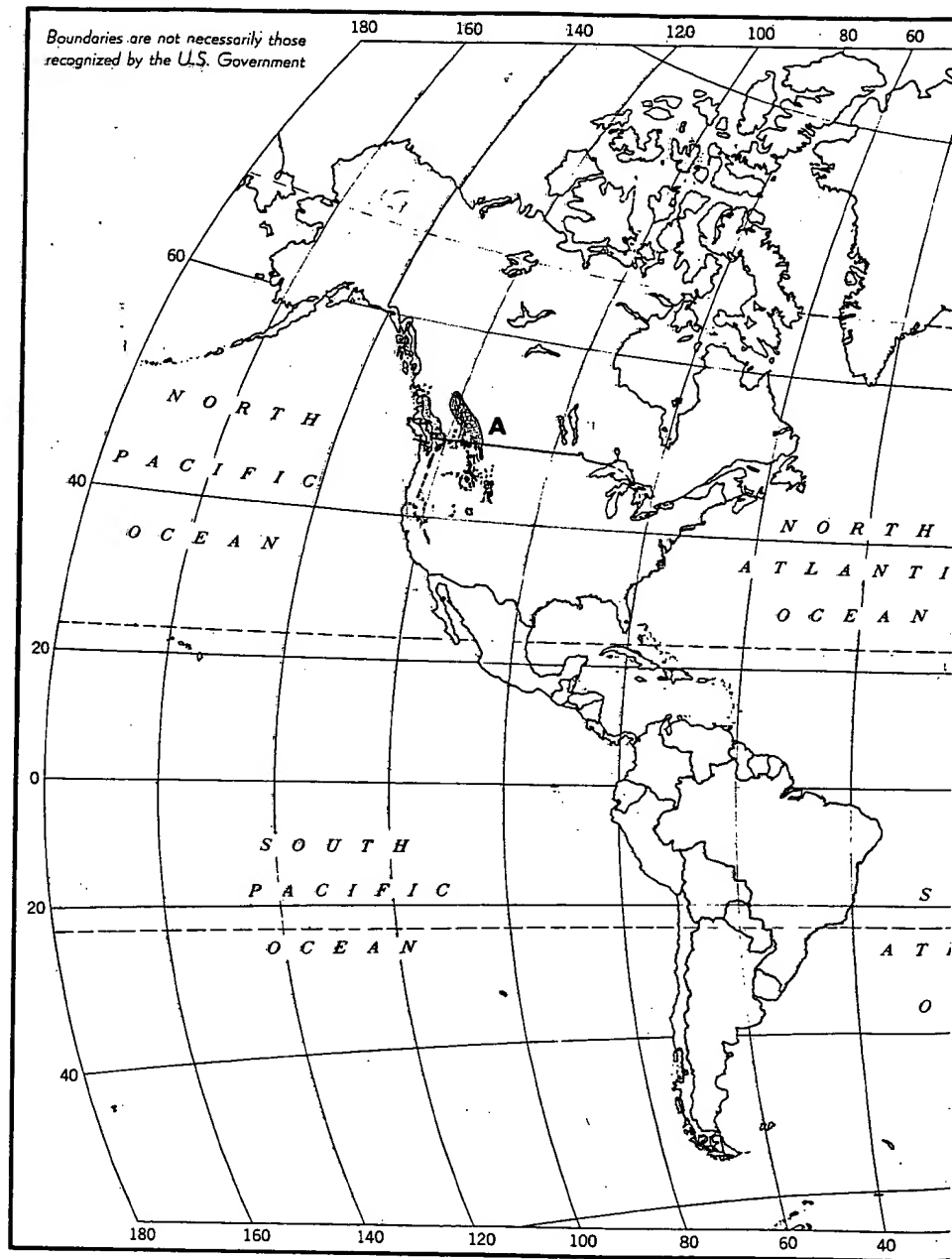


krempfi, K.).

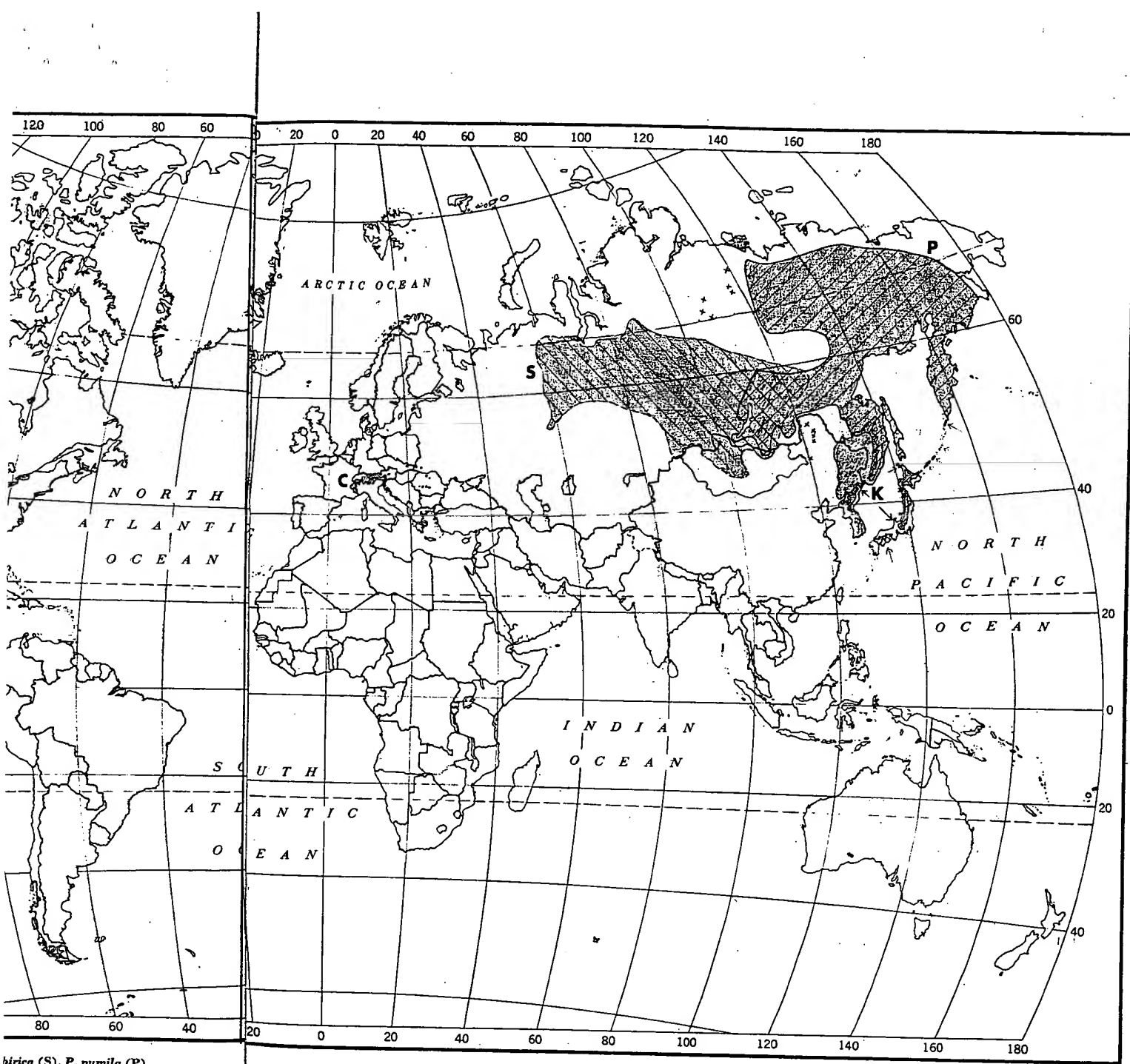


Map 3. *Pinus* subgen. *Pinus* (62 species).

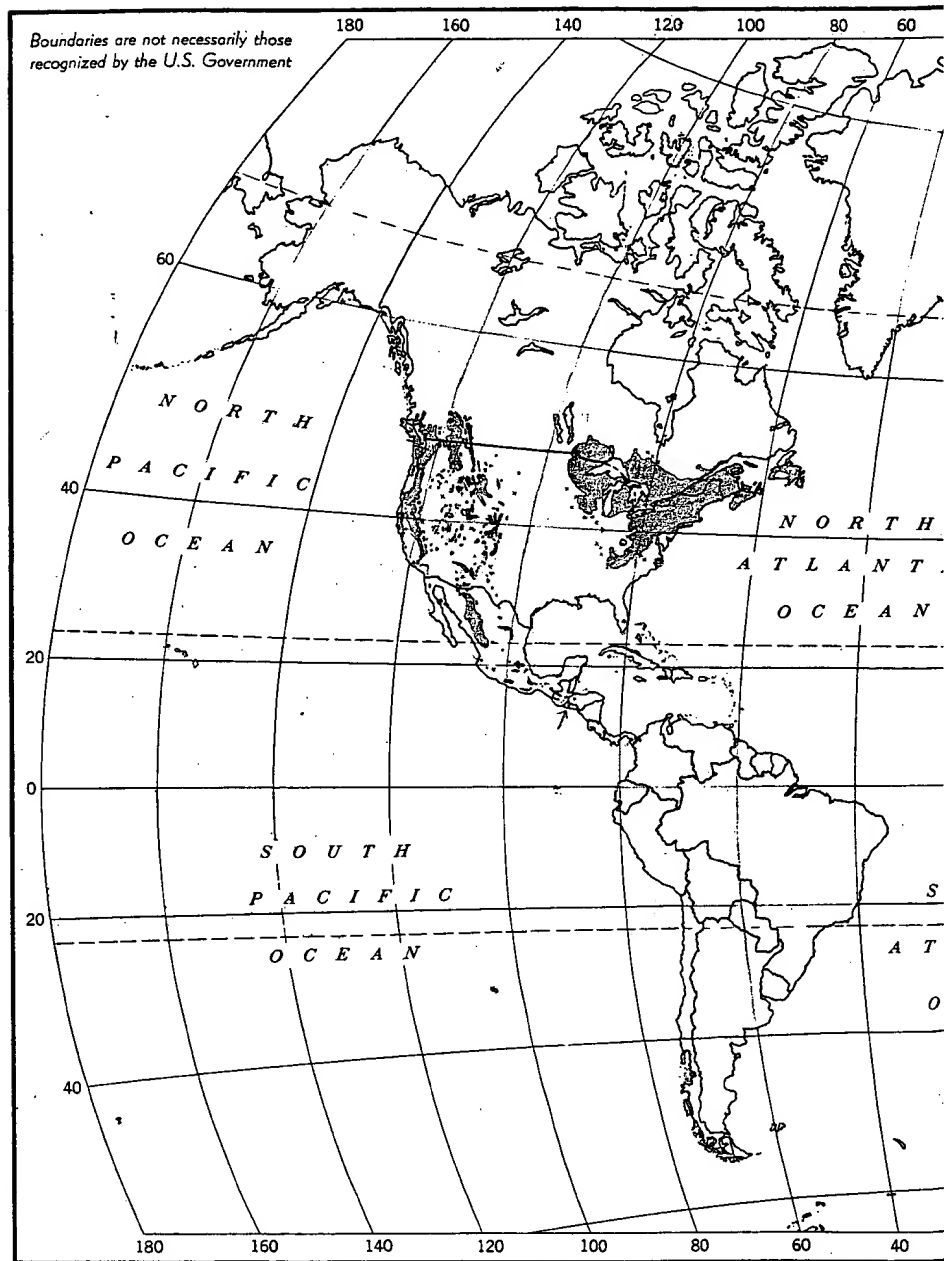




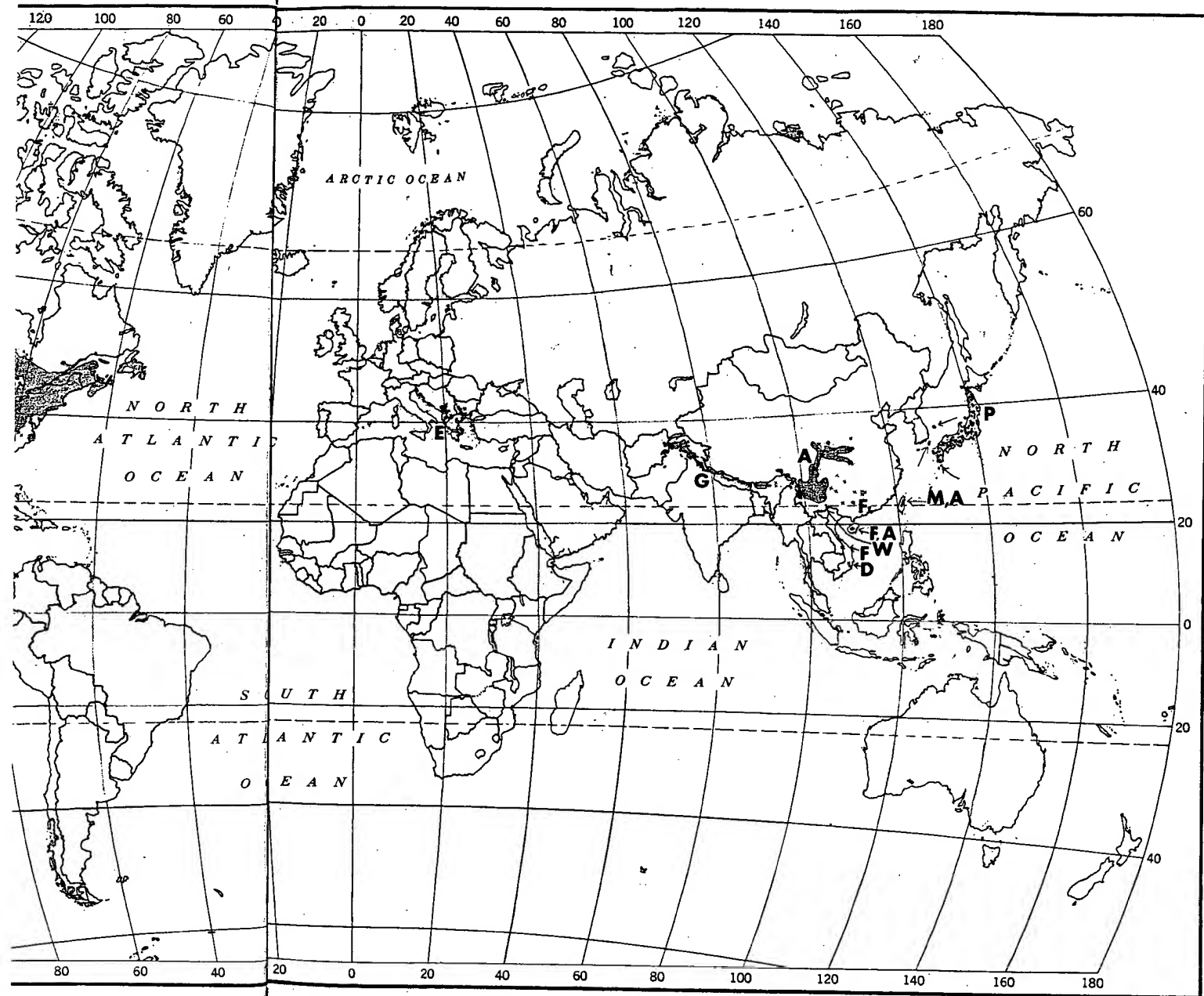
Map. 4. *Pinus* subsect. *Cembrae* (5 species). *P. albicaulis* (A), *P. cembra* (C), *P. sibirica* (S), *P. pumila* (P), *P. koraiensis* (K).



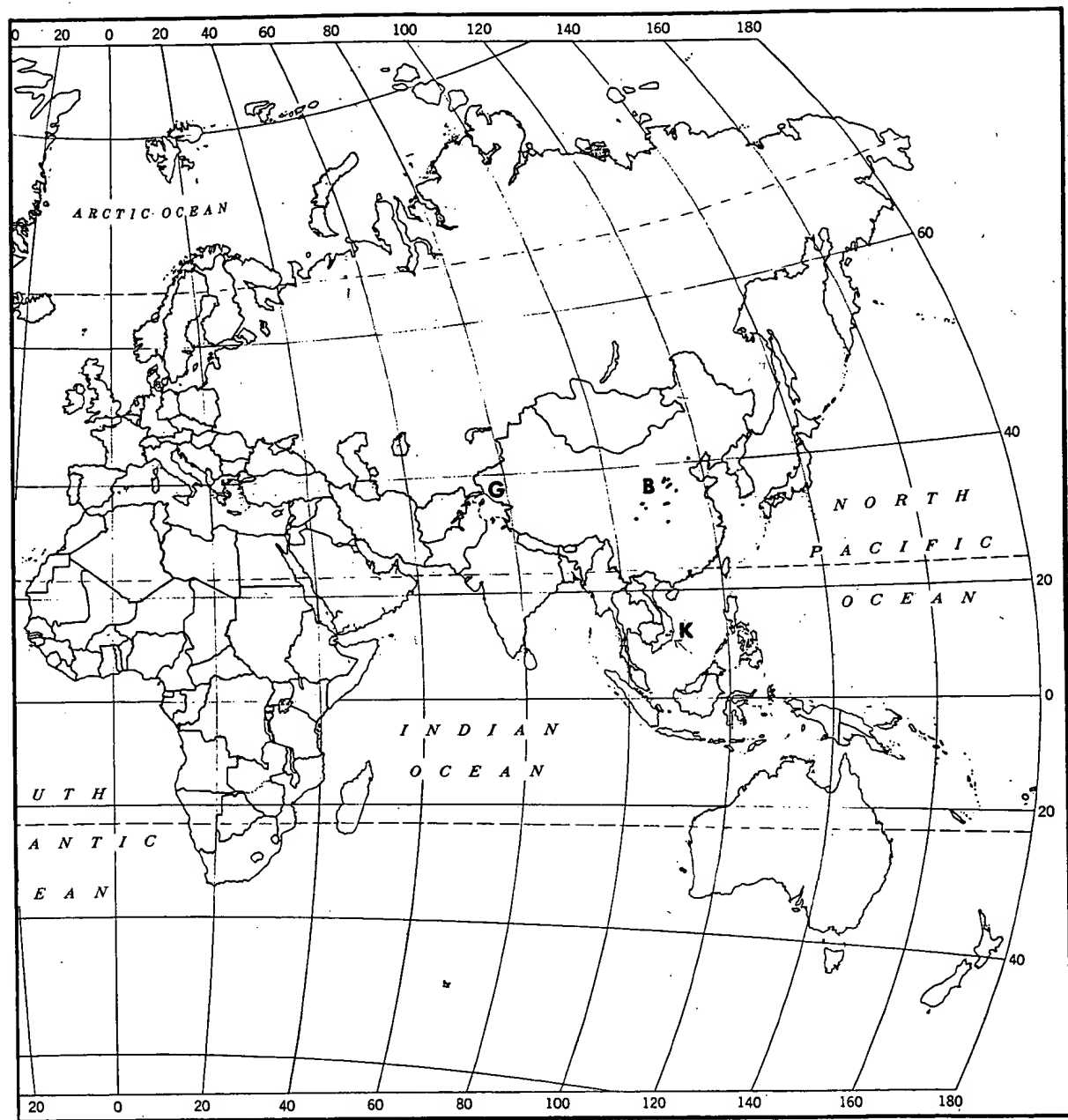
birica (S), *P. pumila* (P),



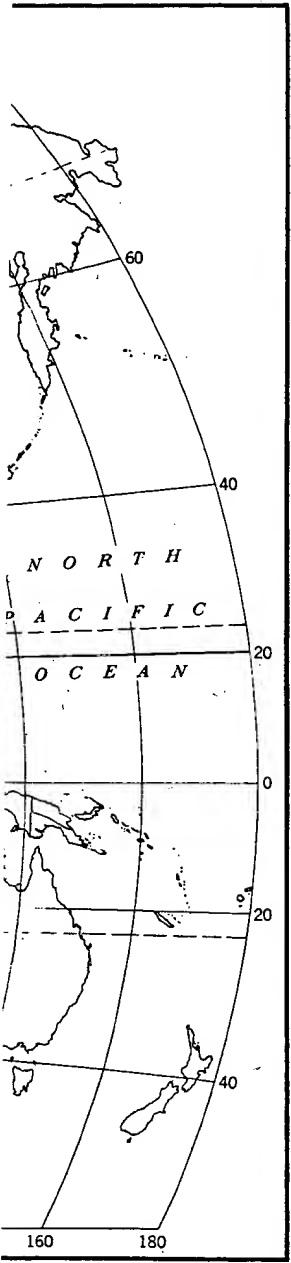
Map. 5. *Pinus* subsect. *Strobi* (14 species). *P. peuce* (E), *P. griffithii* (G), *P. armandii* (A), *P. parviflora* (P), *P. fenzeliana* (F), *P. dalatensis* (D), *P. morrisonicola* (M), *P. wangii* (W).



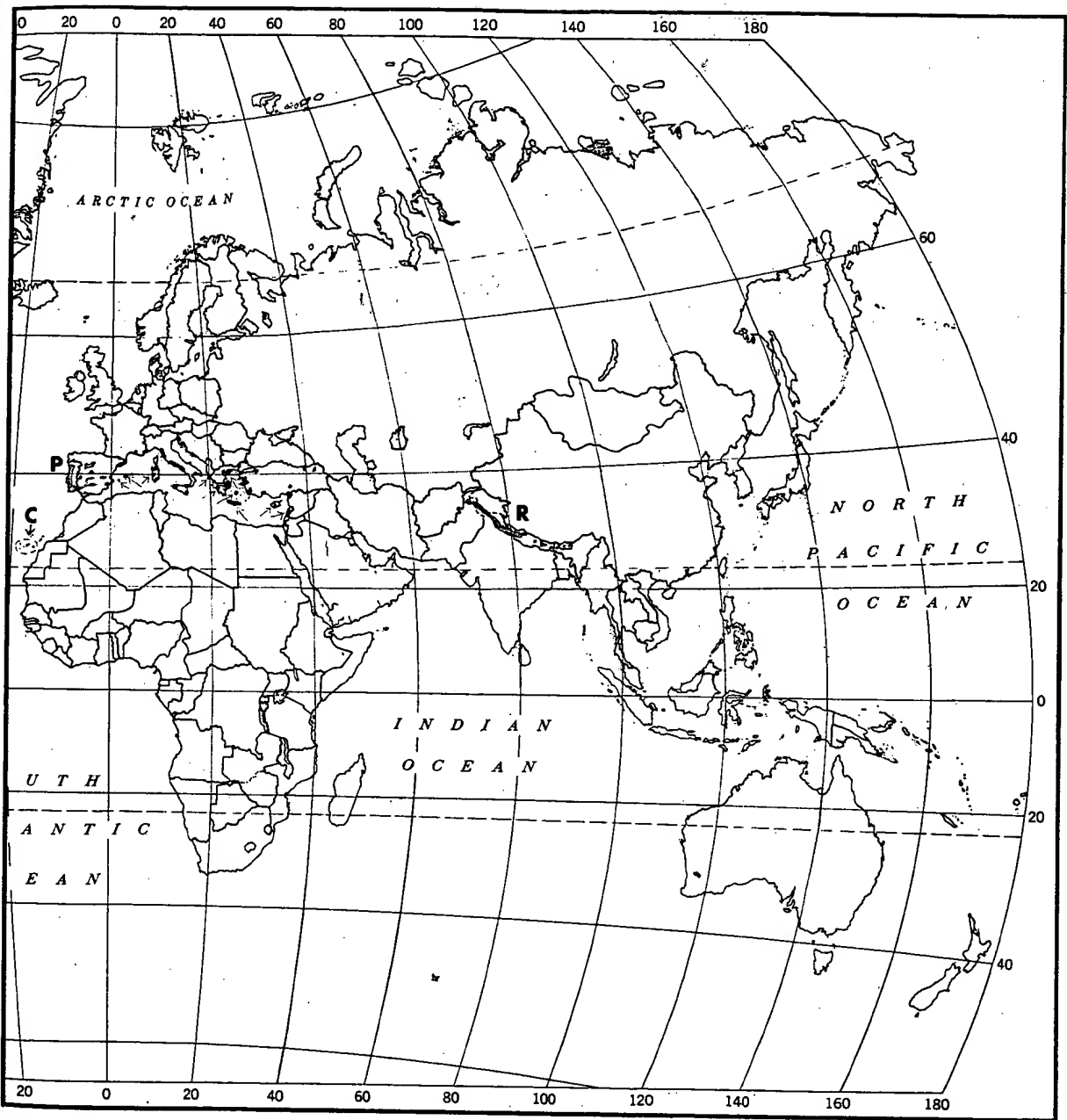
andii (A), *P. parviflora* (P),
 7).



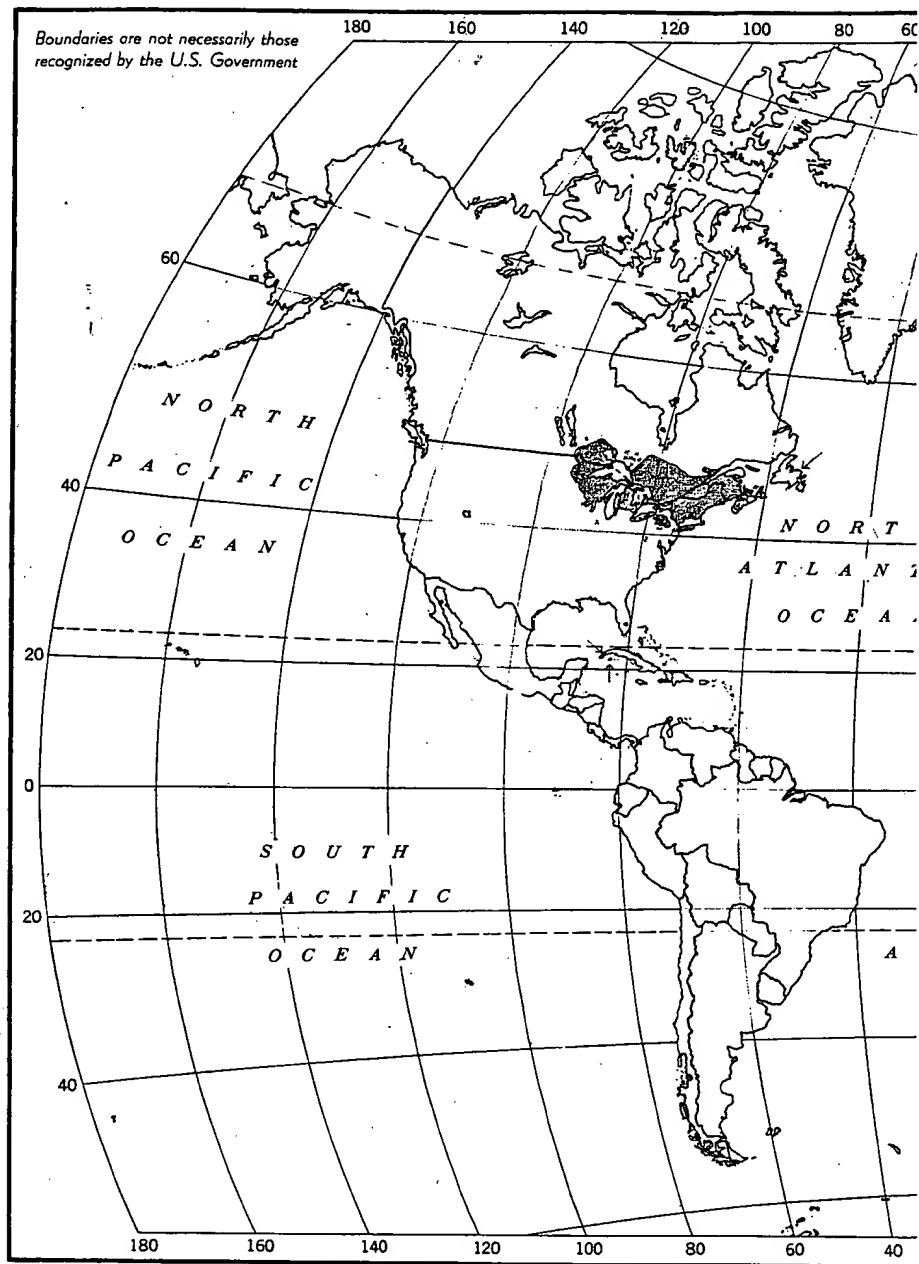
Map 6. *Pinus* subsect. *Gerardianae* (2 species, *P. gerardiana*, G, and *P. bungeana*, B) and subsect. *Krempfianae* (1 species, *P. krempfii*, K).



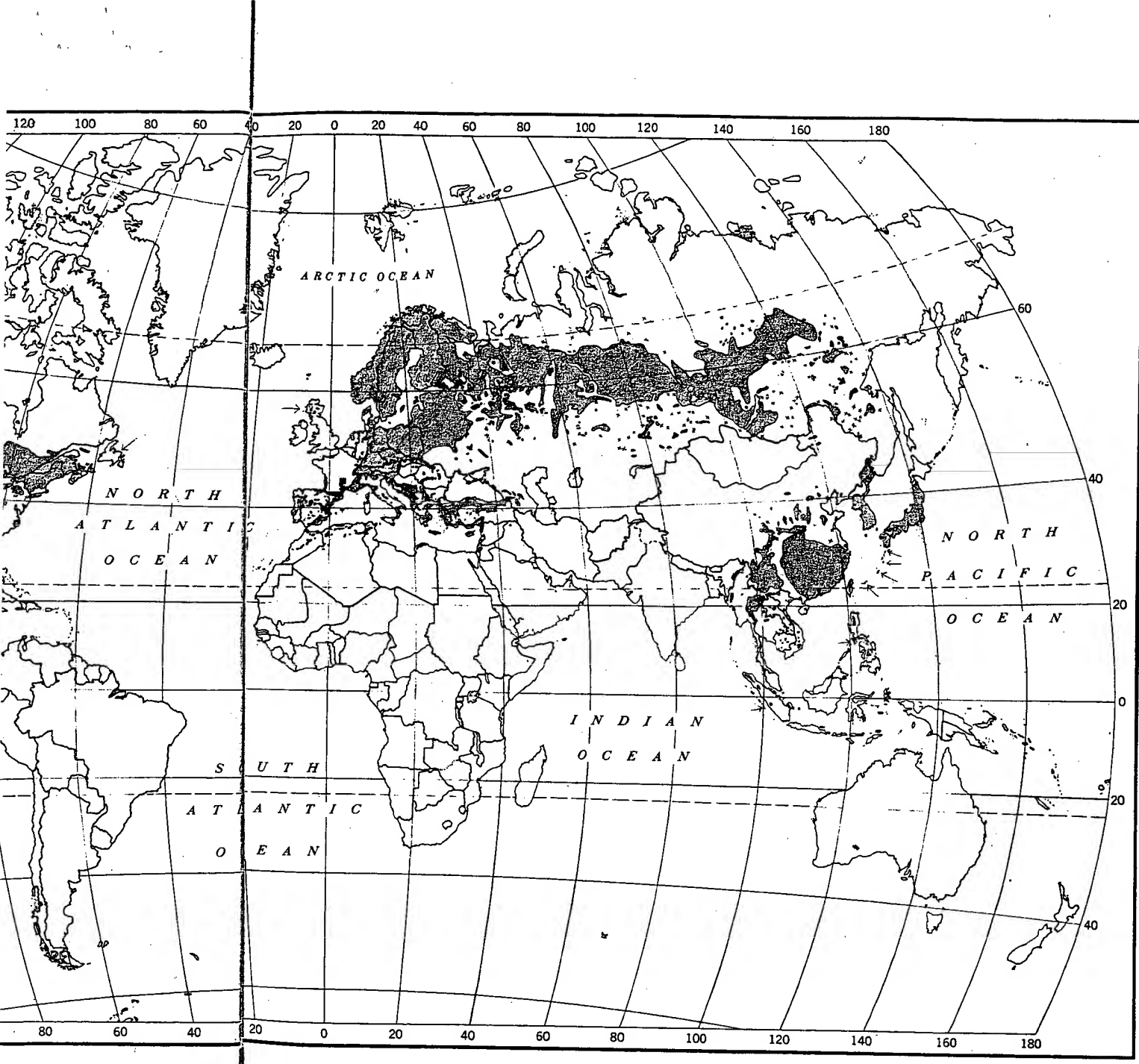
1 species, *P. krempfi*, K).



Map 7. *Pinus* subsect. *Canariensis* (2 species, *P. canariensis*, C, and *P. roxburghii*, R) and subsect. *Pineae* (1 species, *P. pinea*, P).

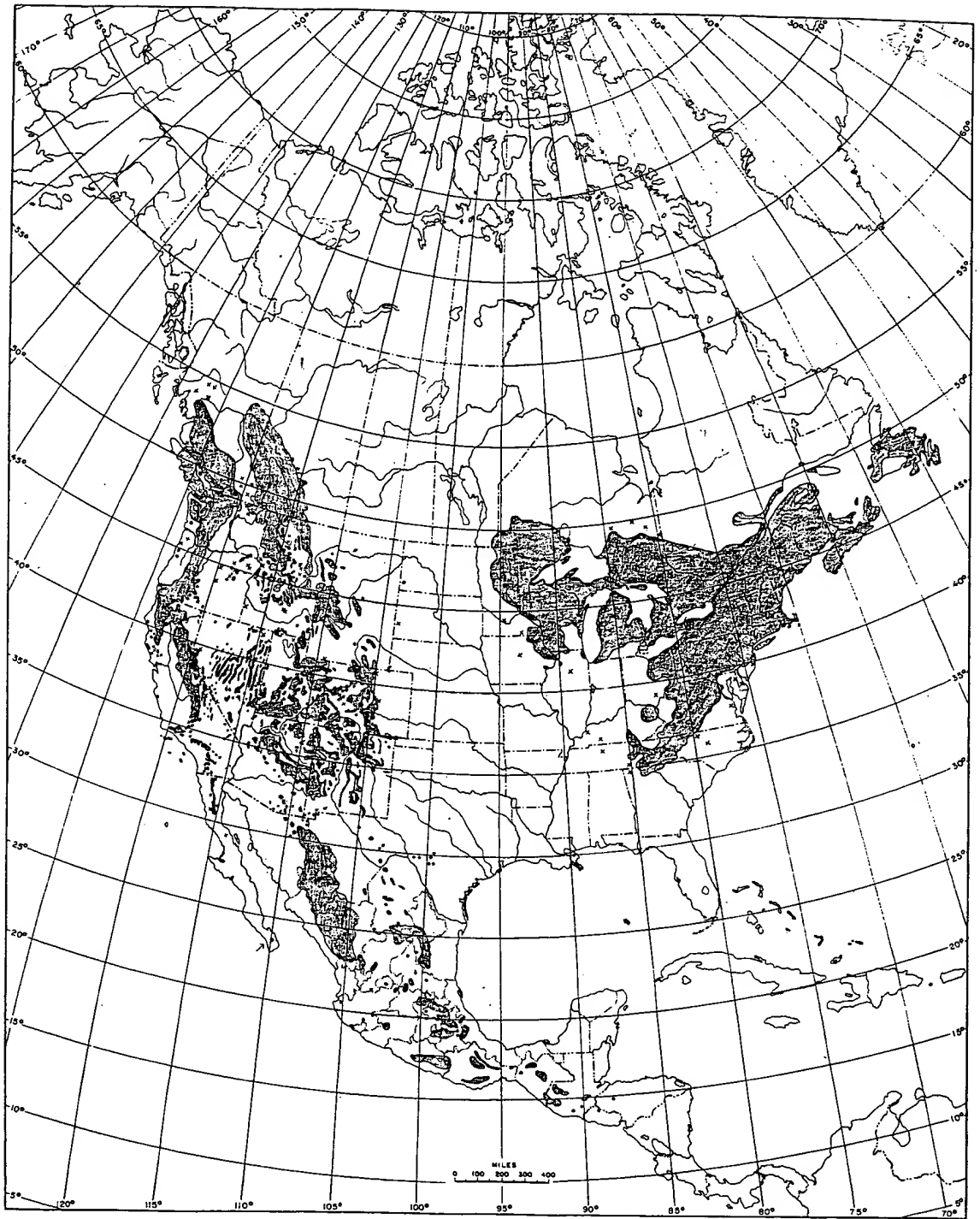


Map 8. *Pinus* subsect. *Sylvestres* (19 species).

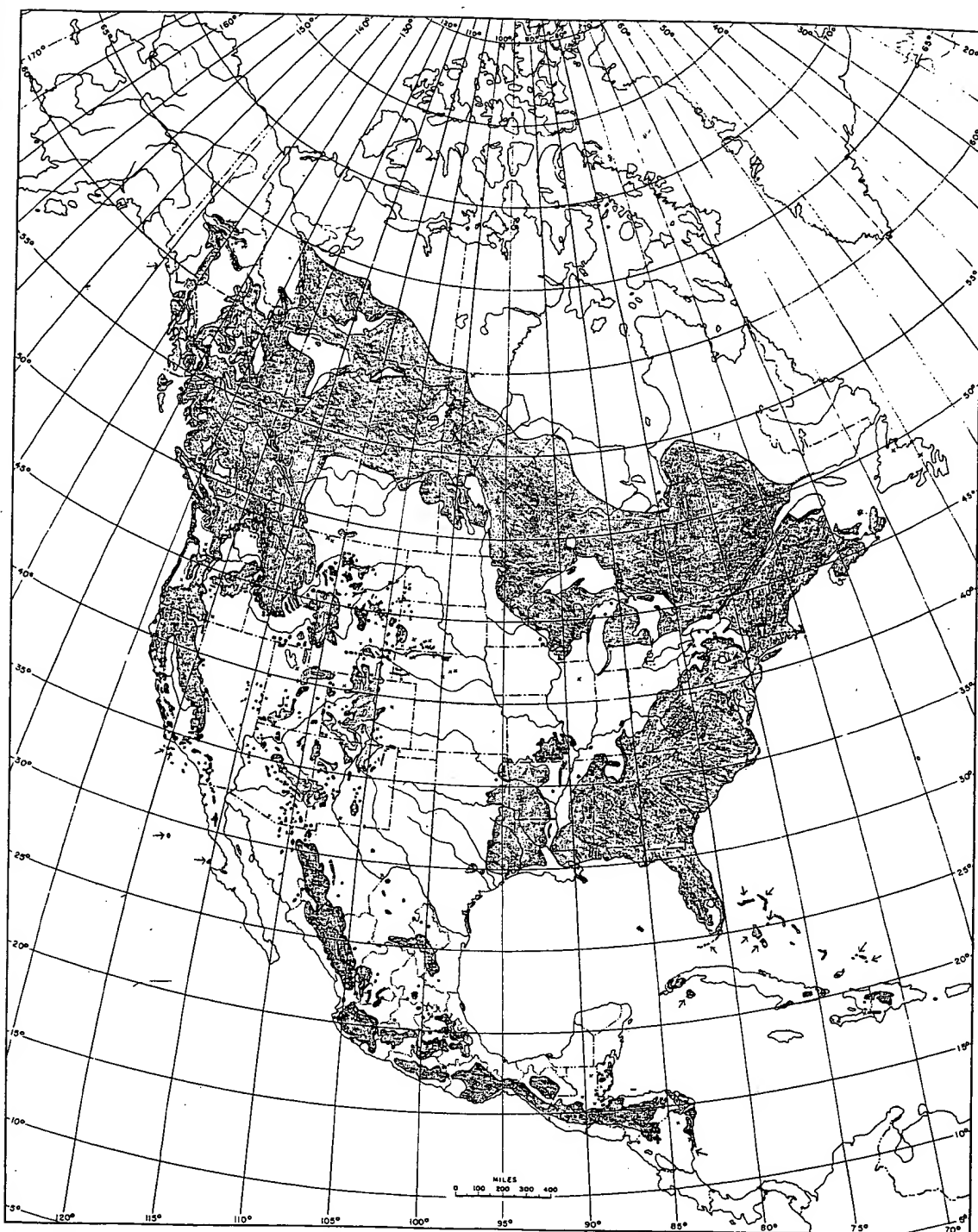
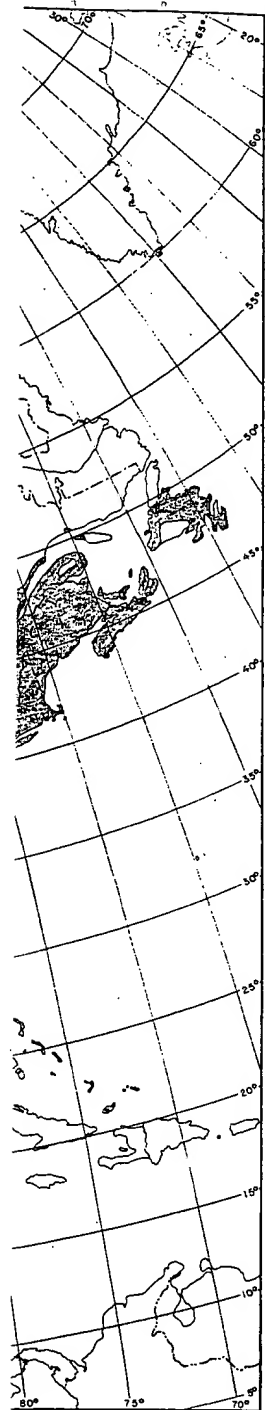




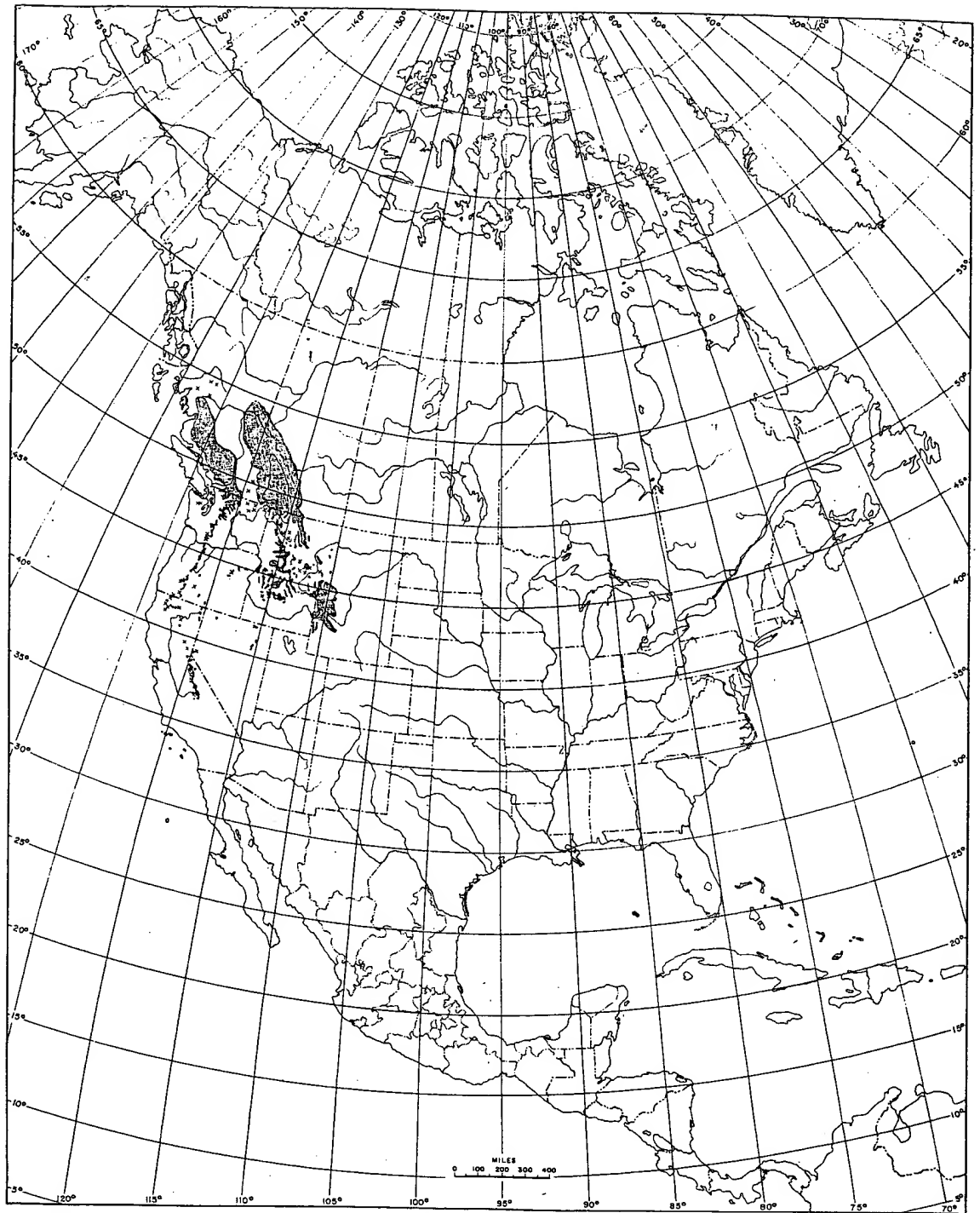
Map 9. The genus *Pinus* in North America (59 species).



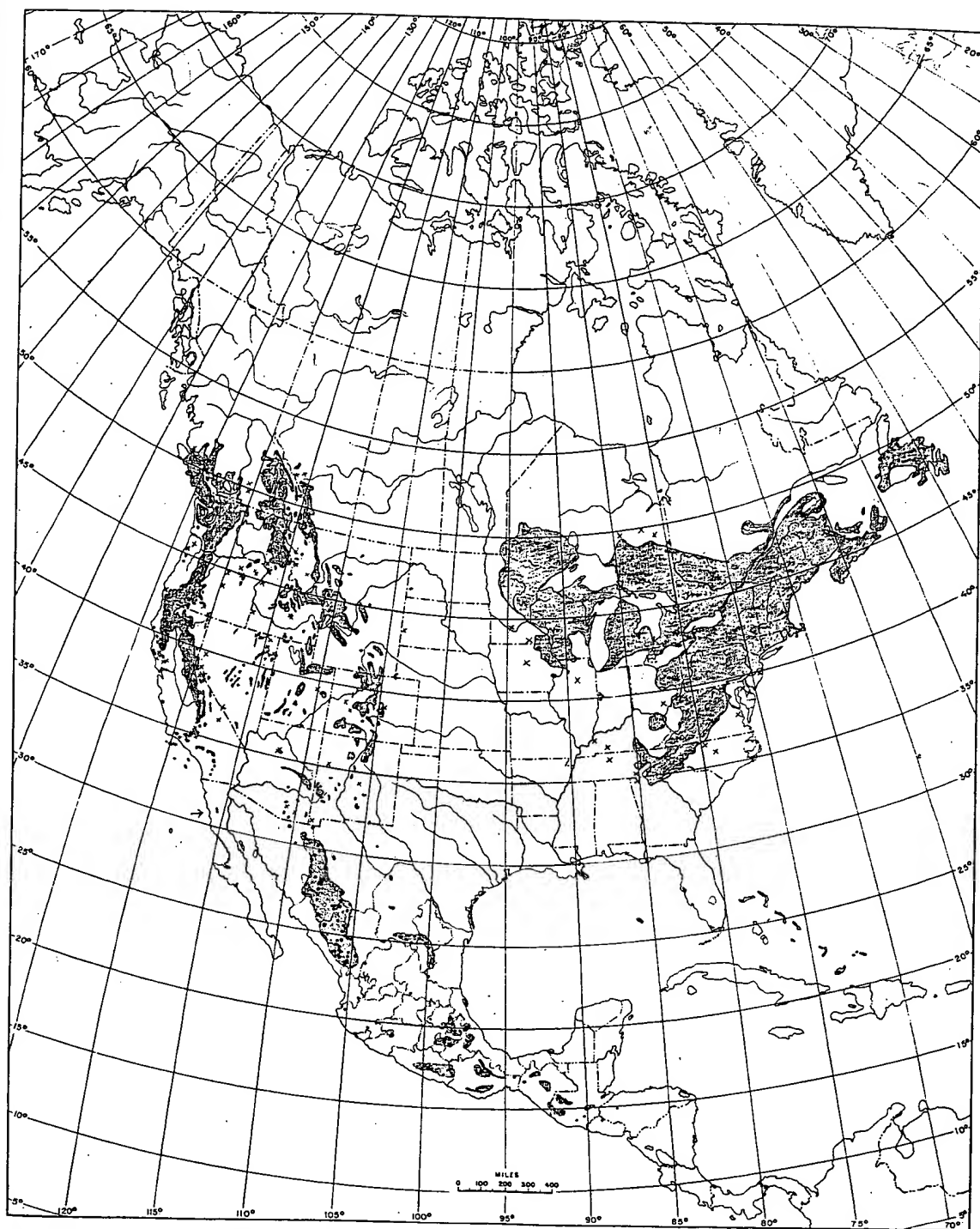
Map 10. *Pinus* subgen. *Strobus* in North America (17 species).



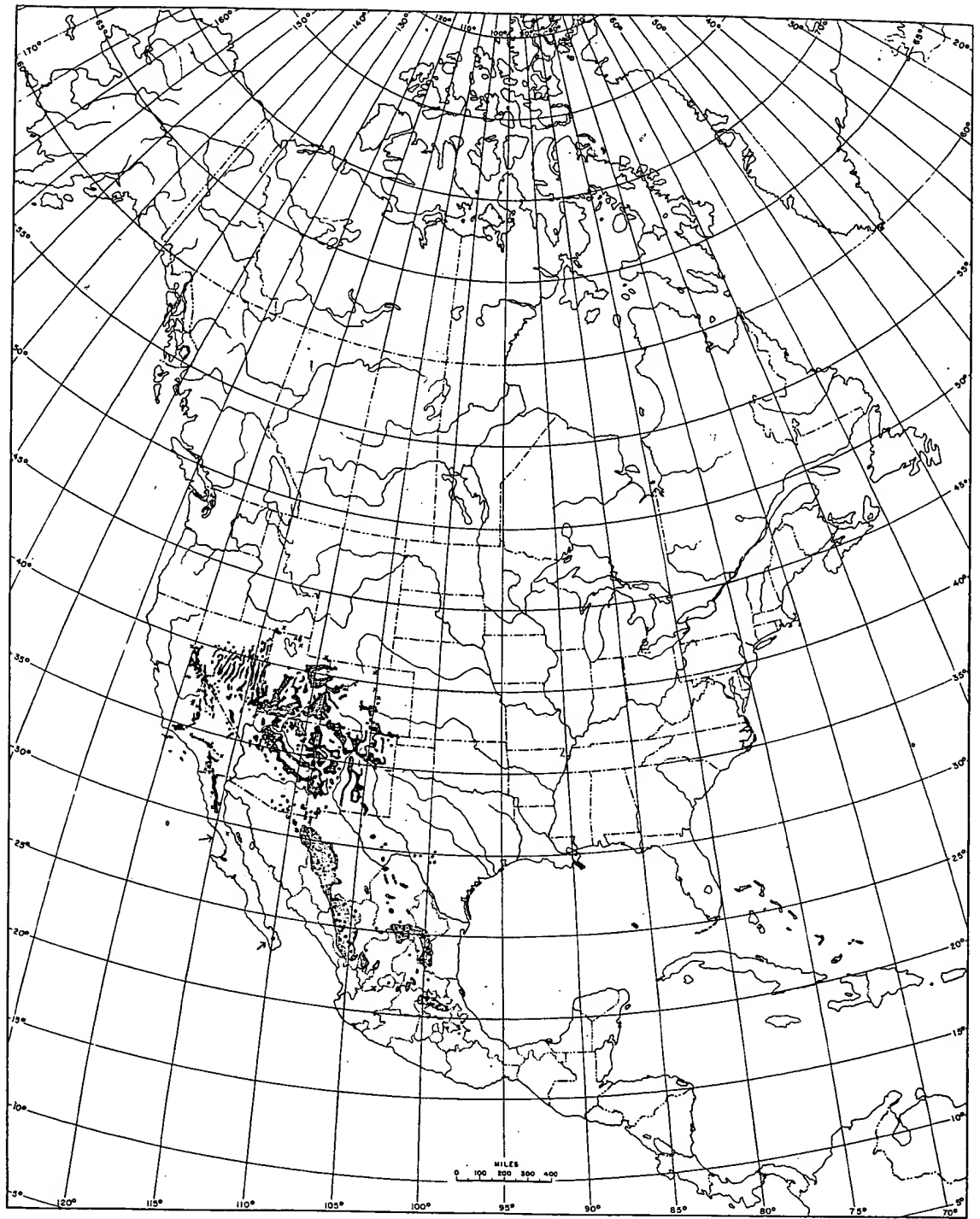
Map 11. *Pinus* subgen. *Pinus* in North America (42 species).



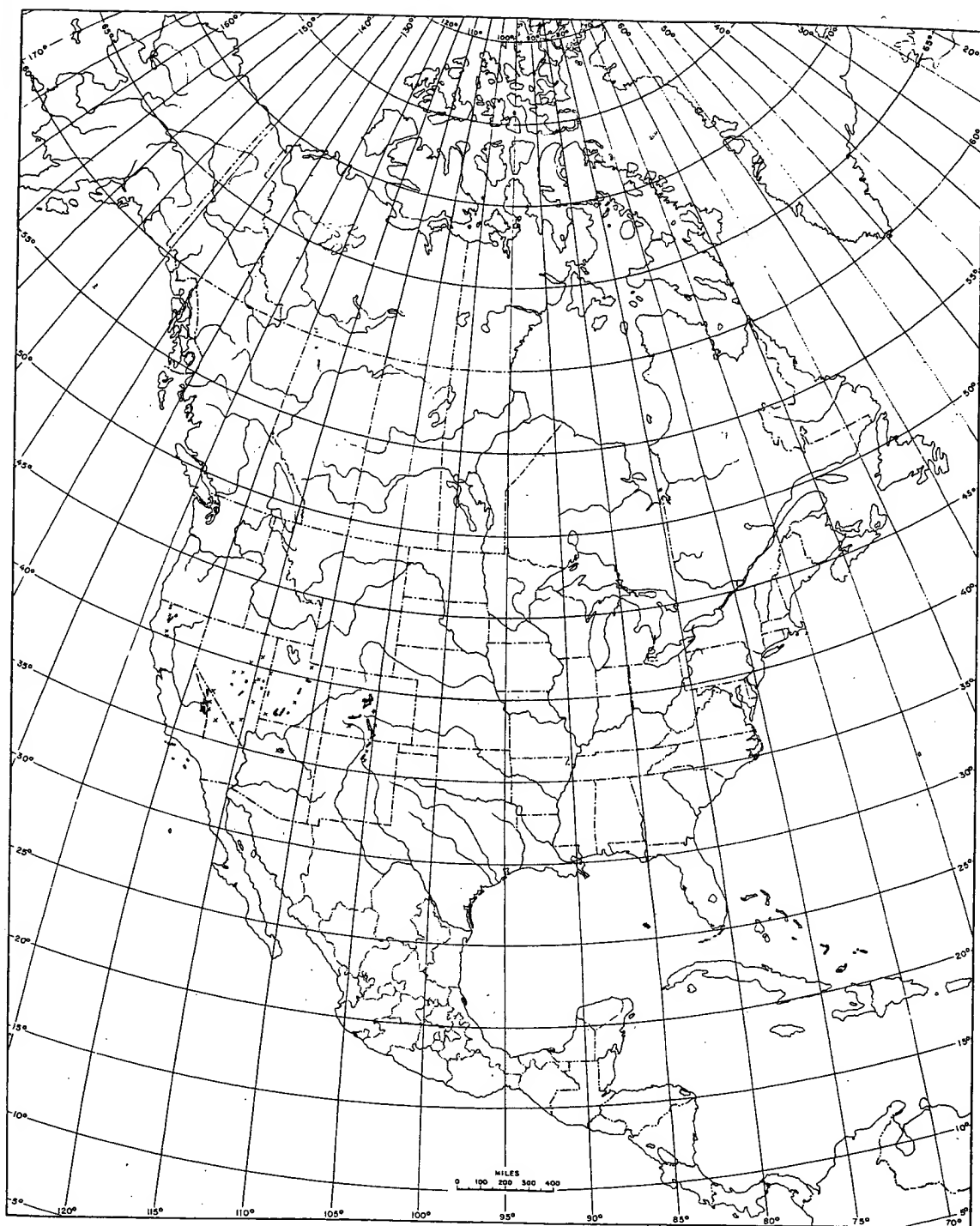
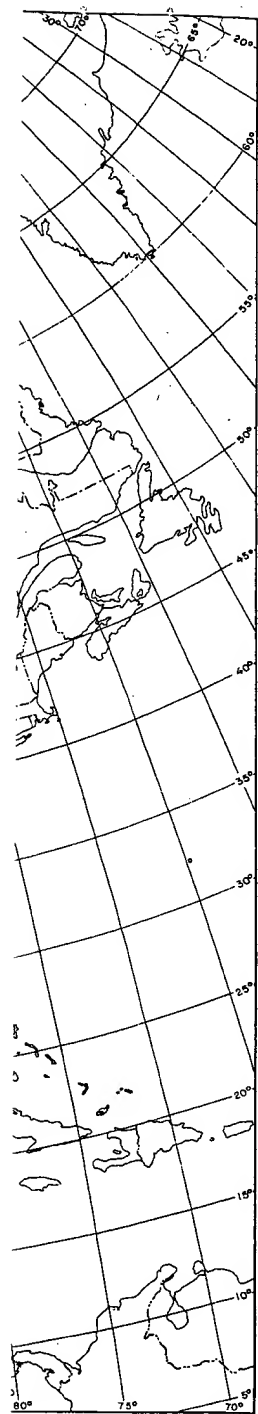
Map 12. *Pinus* subsect. *Cembrae* in North America (1 species, *P. albicaulis*).



Map 13. *Pinus* subject. *Strobi* in North America (6 species).



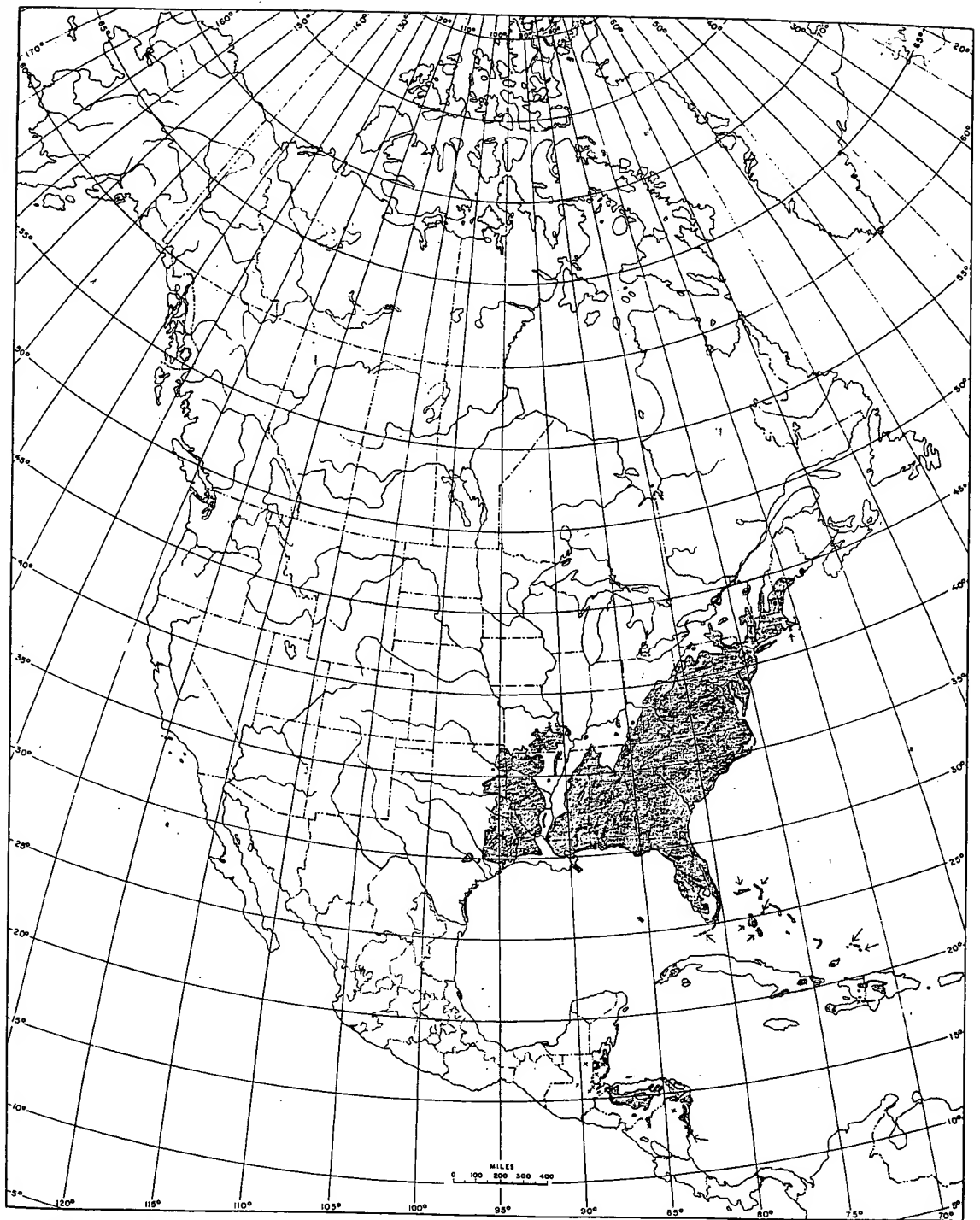
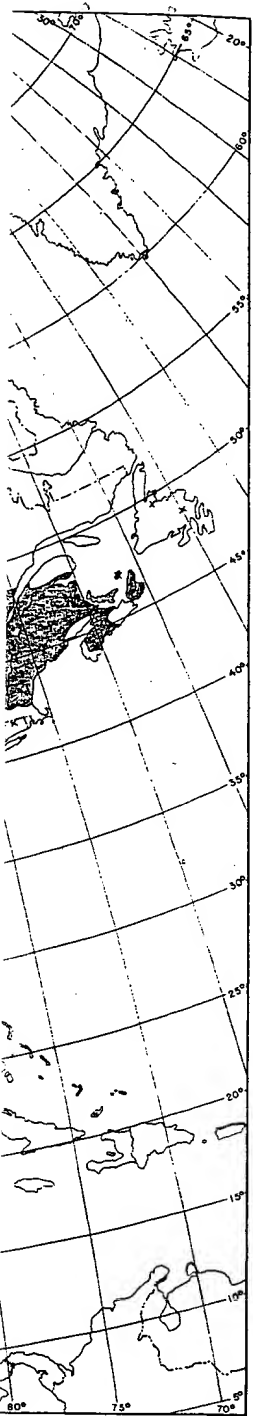
Map 14. *Pinus* subsect. *Cembroides* (8 species).



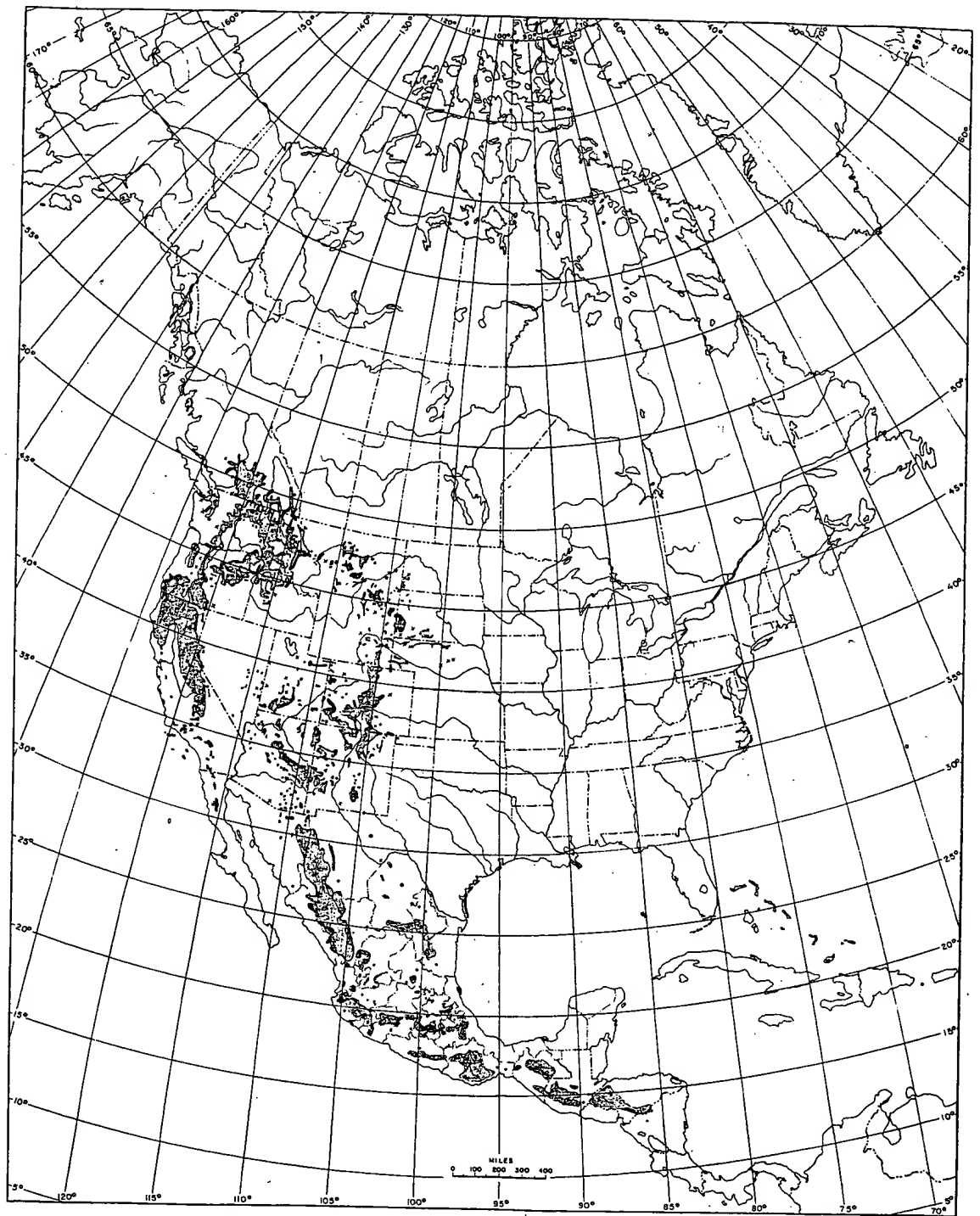
Map 15. *Pinus* subsect. *Balfourianae* (2 species). *P. balfouriana* (California, west of broken line), *P. aristata* (east of broken line).



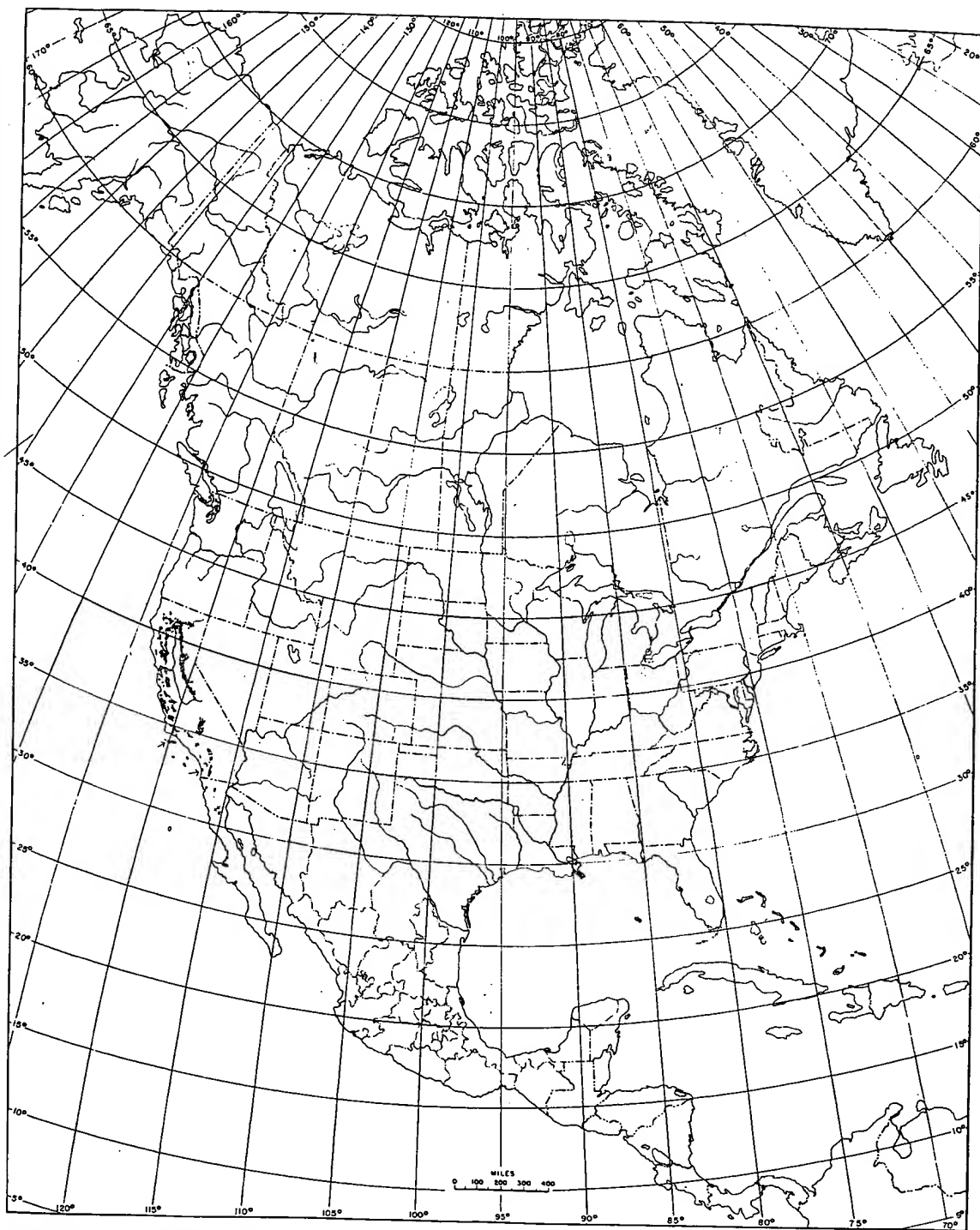
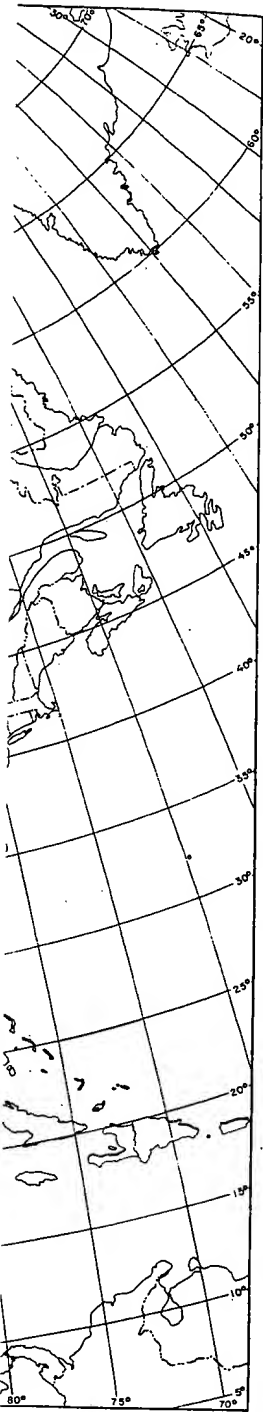
Map 17. *Pinus* subject *Sylvestres* in North America (2 species), *P. resinosa* (northeast) and *P. tropicalis* (Cuba).



Map 18. *Pinus* subsect. *Australes* (11 species).



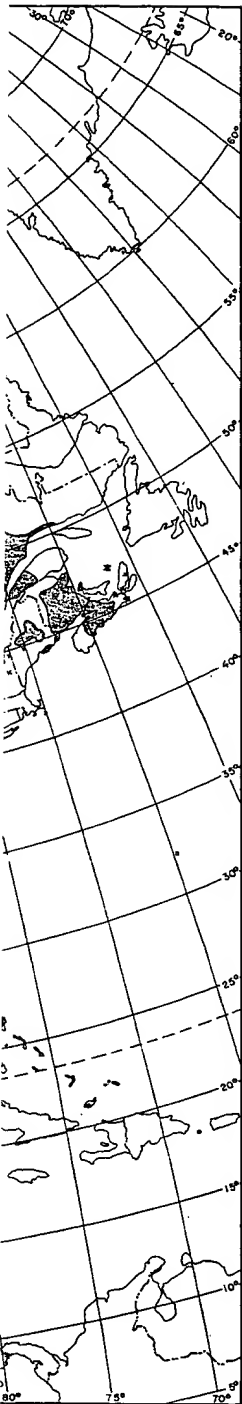
Map 19. *Pinus* subsect. *Ponderosae* (13 species).



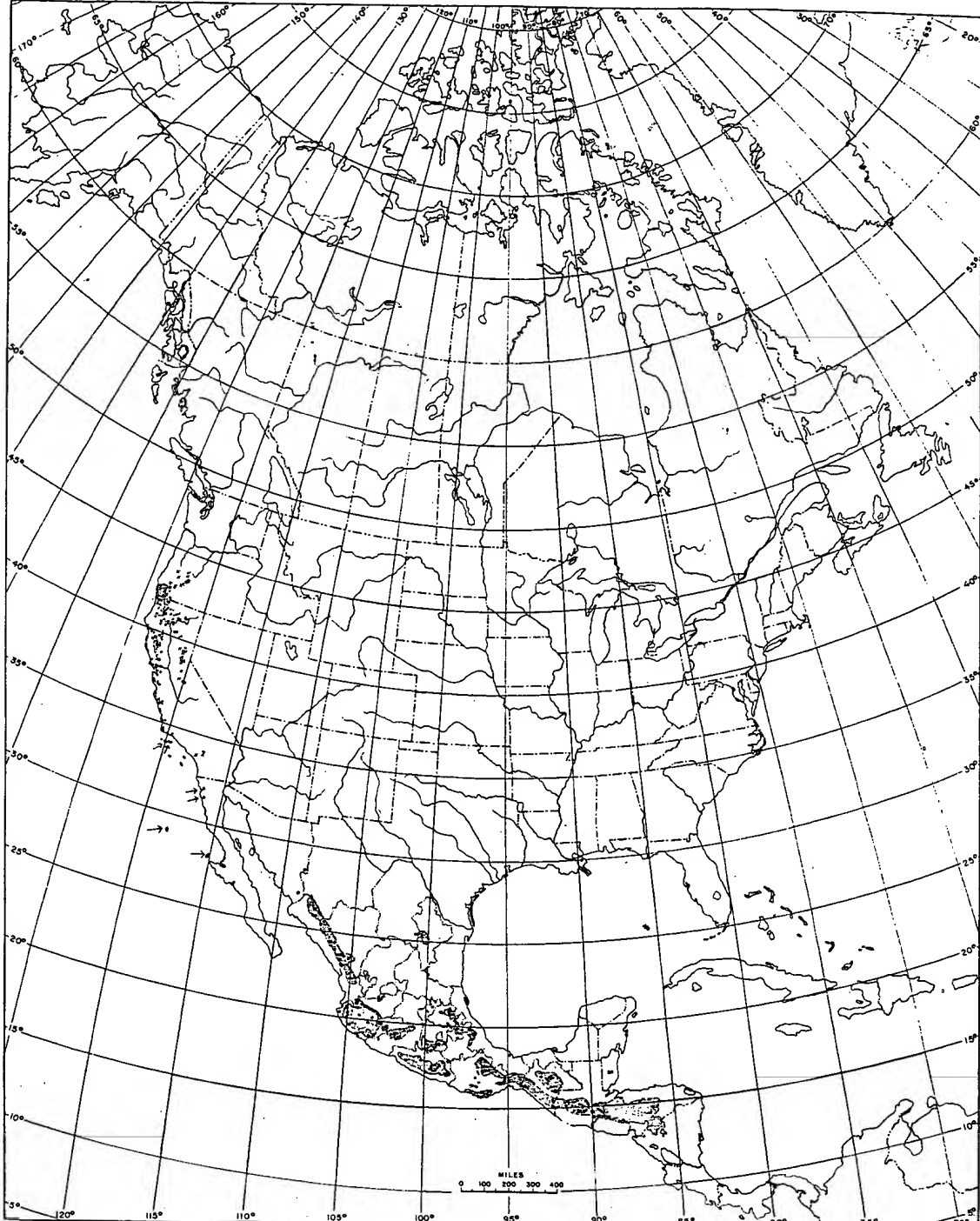
Map 20. *Pinus* subsect. *Sabinianae* (3 species; *P. torreyana*, 2 arrows).



Map 21. *Pinus* subsect. *Contortae* (4 species). *P. contorta* (western, east to dotted line), *P. banksiana* (northern, west to broken line), *P. virginiana* (eastern), *P. clausa* (Florida).



thern, west to broken line),



Map 22. *Pinus* subject. *Oocarpae* (7 species).

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US006200809B1

(12) **United States Patent**
Klimaszewska et al.

(10) Patent No.: **US 6,200,809 B1**
 (45) Date of Patent: **Mar. 13, 2001**

(54) **MATURATION OF SOMATIC EMBRYOS**

(75) Inventors: **Krystyna Klimaszewska; Benjamin C. S. Sutton**, both of Vancouver; **Daniel R. Polonenko**, Coquitlam; **David R. Cyr**, Vancouver; **Thomas F. Stodola**, Burnaby, all of (CA)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/135,264**

(22) Filed: **Aug. 17, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/078,285, filed on Mar. 17, 1998.

(51) Int. Cl.⁷ **C12N 5/00; C12N 5/04; A01H 4/00; A01H 7/00**

(52) U.S. Cl. **435/422; 435/410; 435/420; 435/430.1; 435/431; 800/319**

(58) Field of Search **435/422, 410, 435/420, 430, 430.1, 431; 800/319**

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(List continued on next page.)

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(74) **Attorney, Agent, or Firm**—Robert H. Barrigar; Barrigar Intellectual Property Group

(57) **ABSTRACT**

A method of developing and maturing somatic embryos in a growth environment, which method comprises manipulating the water availability of the growth environment using a physical means of control. The invention also provides a growth environment for maturing somatic embryos, wherein the water potential of the embryogenic tissue is manipulated to optimize somatic embryo development and maturation. The invention further relates to a somatic embryo matured by the method of the invention. In the invention, a physical means of control is used to affect the water potential of the embryogenic tissue and developing somatic embryos growth medium, rather than a chemical means such as the introduction of PEG, to stimulate the maturation of the embryos. The physical means may be operated, for example, by separating the somatic embryos from the growth medium by a porous support, or by introducing a gelling agent (e.g. gellan gum) into the growth medium in larger than normal quantities.

24 Claims, 2 Drawing Sheets

Exhibit 4
 Connett '088

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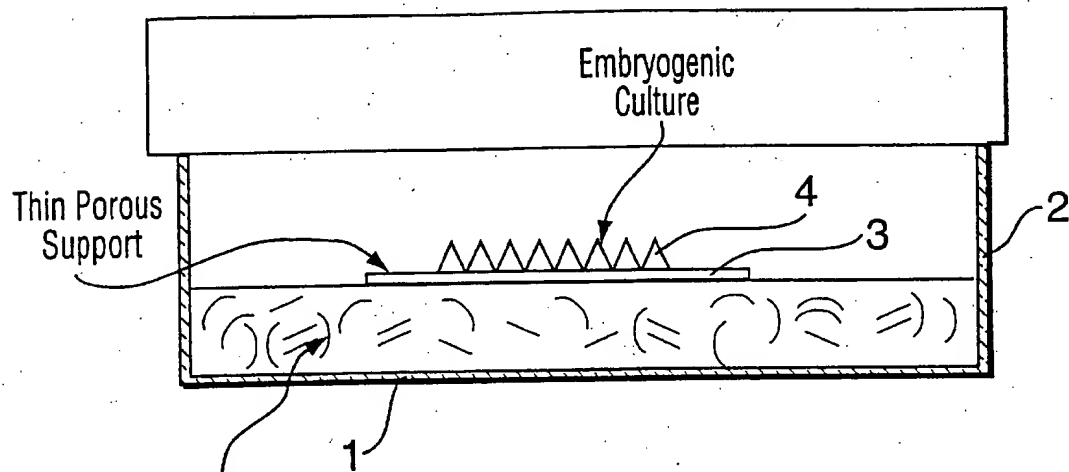


FIG. 1a

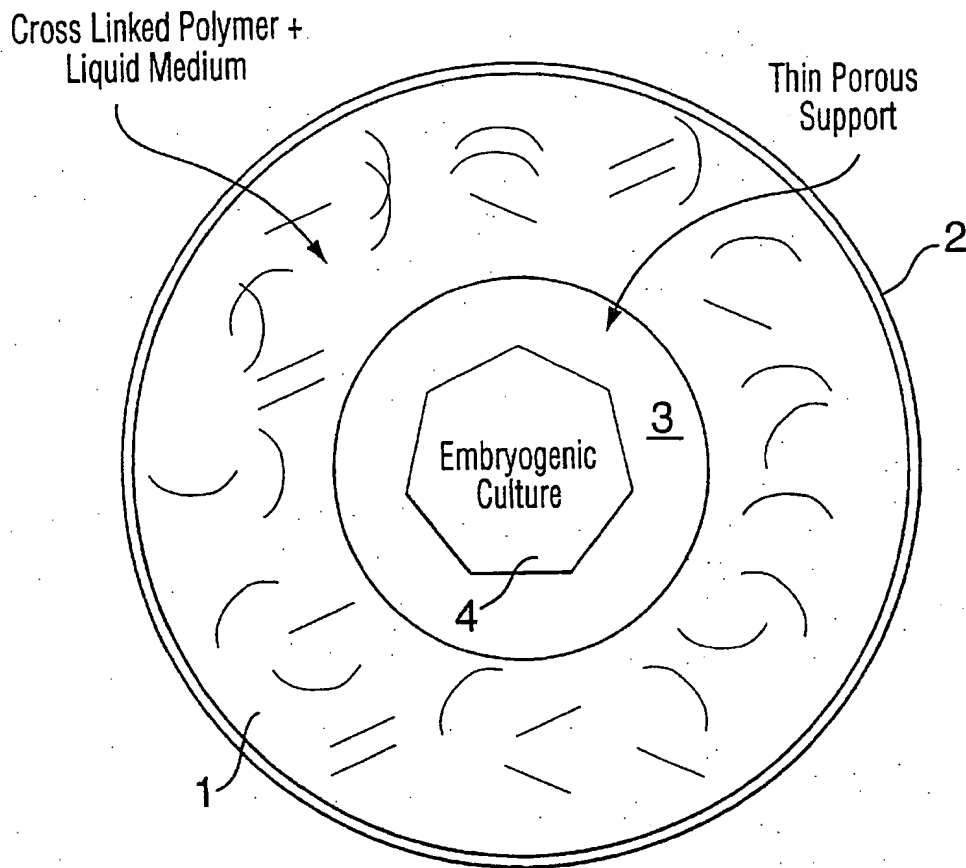
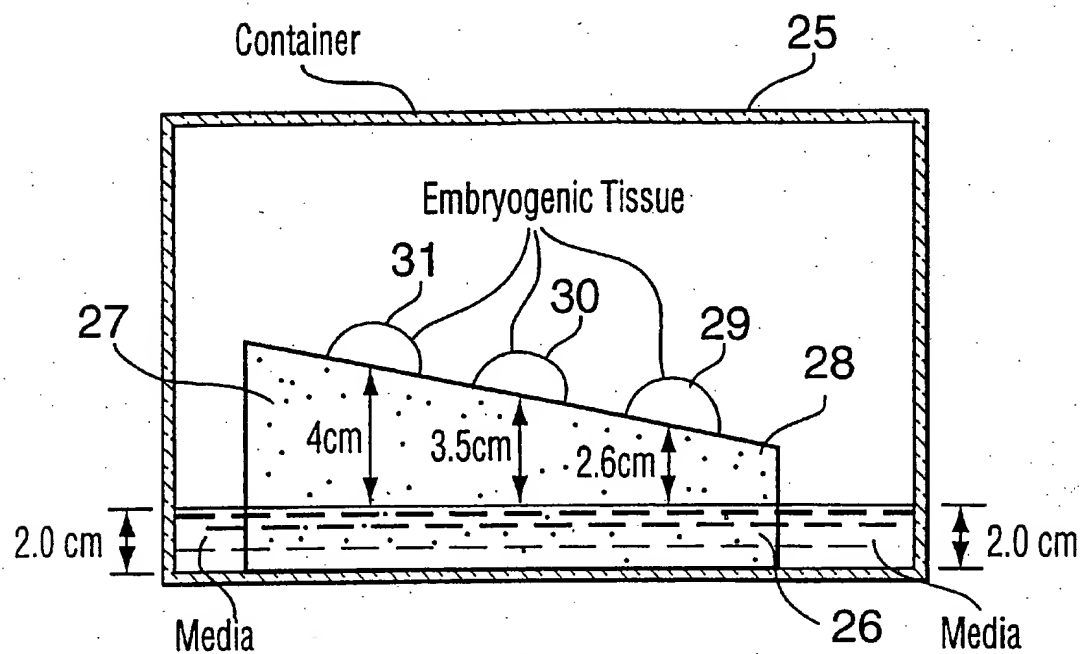
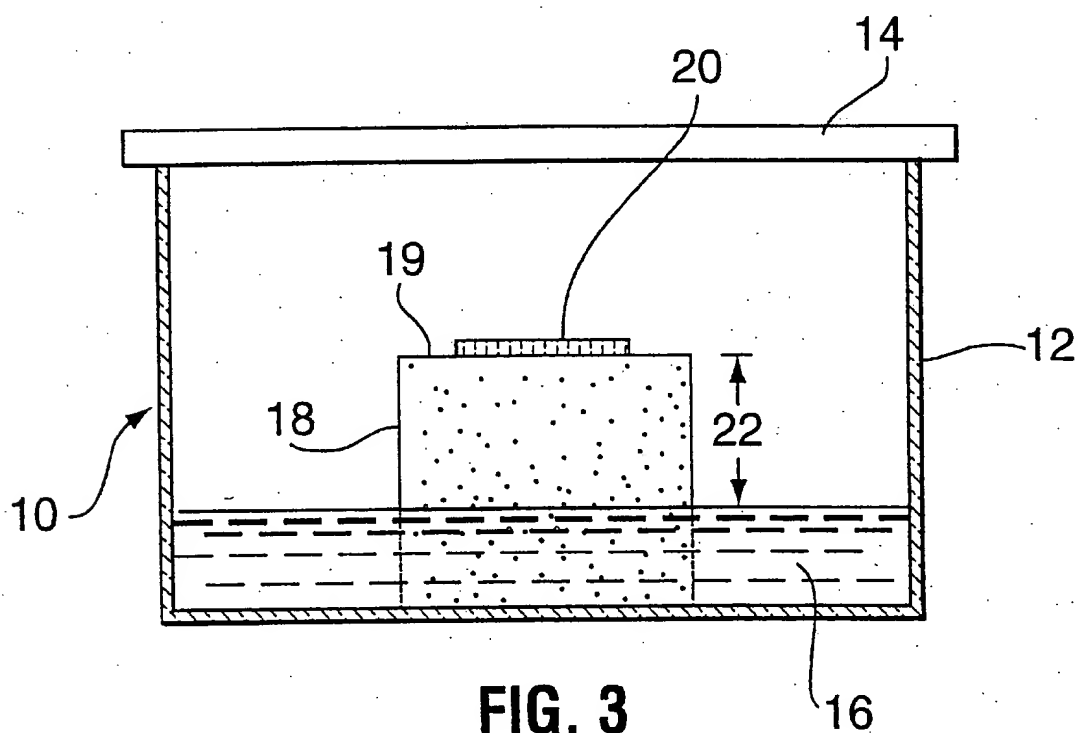


FIG. 1b

**FIG. 2****FIG. 3**

MATURATION OF SOMATIC EMBRYOS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is related to our prior provisional patent application Ser. No. 60/078,285 filed Mar. 17, 1998.

BACKGROUND OF THE INVENTION

The present invention relates to the field of somatic embryogenesis and, in particular, to methods of manipulating the maturation of somatic embryos within culture vessels.

Recently, somatic embryogenesis has gained attention as it offers a possible low-cost means for clonal reproduction of large numbers of plants of various species. The steps of somatic embryogenesis, including the initiation and proliferation of embryogenic cultures from explant tissues, have been documented in the art for many plants, including angiosperms and gymnosperms. Simply, the method of somatic embryogenesis involves the selection of an explant of a desired plant. The explant is removed from the parent plant tissue by excision and then subsequently cultured on at least one medium to produce a cell mass capable of further differentiation or development. The cell mass can be maintained and proliferated in the undifferentiated state indefinitely, or manipulated to stimulate differentiation into immature somatic embryo structures which can then be further cultured to form mature somatic embryos. Mature somatic embryos can be harvested and germinated immediately, or dried and then germinated, or dried and stored until required for germination.

Somatic embryos are known to be stimulated to develop and mature in culture if environmental stresses are imposed, such as heat, nutrient depletion, solute-based water stress or increased levels of the plant hormone abscisic acid ("ABA"), whether added exogenously or induced endogenously (see U.S. Pat. No. 5,238,835 to McKersie et al., the contents of which are incorporated herein by reference, said patent referred to hereinafter as the McKersie patent). The McKersie patent discloses the use of stress, including osmotic, nutrient, water and heat stresses among others, to trigger the endogenous production of ABA within somatic embryogenic cultures.

Due to the fact that somatic embryos develop without the surrounding nutritive tissues, i.e. megagametophytes in gymnosperm species and endosperm in angiosperm species, and protective seed coats normally present in zygotic seeds, research has focused on comparing the types and quantities of storage reserves (e.g. lipids, proteins, amino acids, monosaccharides and polysaccharides) produced in somatic embryos with those (average levels) in zygotic seeds of the same species, and on assessing their potential for improving the ease of handling, storage stability, and germination vigour of somatic embryos. Exogenous applications of ABA, and solutes such as polyethylene glycol ("PEG"—most commonly having a molecular weight of 4,000, but possibly ranging in molecular weight from 2,000 to 8,000) have been proposed as useful adjuncts for enhancing the levels of storage reserves in plant cells and in particular, somatic embryos. Specifically, it has been shown that ABA or PEG can be used to promote or otherwise enhance the maturation step of the somatic embryogenesis process with gymnosperms, e.g. conifers, and to reduce the occurrence of precocious germination during the maturation step (Roberts et al. 1990; Attree et al. 1991; Flinn et al. 1991; Carrier et al. 1997). The embryos which result from PEG and/or ABA

facilitated maturation may be larger than their zygotic counterparts and may exhibit greater storage protein and lipid reserves (Flinn et al., 1991; 1995 U.S. Pat. No. 5,464,769 to Attree & Fowke, the contents of which are incorporated herein by reference, and said patent referred to herein as the Attree patent). Conifer somatic embryos produced on media containing PEG and having enhanced lipid levels and reduced moisture contents have been disclosed (the Attree patent). The use of ABA-amended media for the production of conifer somatic embryos with these same attributes have also been previously disclosed (Flinn et al. 1991; Carrier et al. 1997).

Accordingly, it is well known to increase the solute concentration in embryogenic culture media by the incorporation of permeating osmotica (i.e. sugars such as sucrose, mannitol or salts). However, there are problems inherent in these agents being absorbed by the symplast of the plant cells which leads to the development of atypical and poorly germinating embryo products. The alternative is to incorporate into the culture media, non-permeating high-molecular-weight compounds such as PEG or dextran (the Attree Patent). However, it has been recently disclosed that non-permeating high-molecular-weight solutes such as PEG and dextran do not reliably produce viable and useful embryos for all conifer species (Find et al., 1997; Klimaszewska & Smith, 1997). It has also been disclosed that, contrary to common belief, small amounts of high molecular weight PEG (8000) enters the cell protoplast or alternatively, bind to the plasmalemma of *Pinus taeda* and sorghum callus cells when cultured on medium containing PEG (Newton et al., 1990). As well, concerns were raised about the adverse action of some unknown organic impurities in commercial PEG sources in the cellular metabolic processes (Plant and Federman, 1985).

A large group of patents held by the Weyerhaeuser Corporation discloses altering the osmotic potential of the medium during maturation of conifer somatic embryos using solutes. One representative patent is U.S. Pat. No. 5,563,061 which describes a multi-phase culturing process in which differently "tailored" media are used at each phase of somatic embryogenesis. During the second and third phases, the early stage embryos are grown for a defined time period on a culture medium containing a higher osmolality than that used in the induction phase. The osmotic potentials in the phase-two and phase-three media are altered by the incorporation of solutes such as sugars, PEG, sorbitol, myoinositol, mannitol, and lactose.

Although the use of PEG or other similar non-permeating solutes discussed above as well as others known in the art, have been used successfully to mimic the chemical, hormonal, and environmental triggers of maturation in producing mature somatic embryos for some plant species including conifers, a large proportion of the embryos produced are atypical and not useful for germination and further propagation. Therefore, it is desirable to avoid the use of PEG and other similar non-permeating solutes for somatic embryogenesis with conifers generally, and with spruce and pine species in particular.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to obviate or mitigate the above disadvantages.

Another object of the invention is to produce high numbers of high-quality plant somatic embryos capable of germination and subsequent conversion to complete and fully functional plants.

A further object of the invention is to minimize the production of unacceptable atypical plant somatic embryos. The present invention provides a method of developing and maturing somatic embryos in a growth environment having a water potential, which method comprises exposing an embryogenic culture of embryogenic tissue or developing and maturing embryos to an aqueous liquid maturation medium, and allowing said embryogenic culture to develop into mature somatic embryos, wherein a physical means is used to affect the availability of water in the growth environment for uptake by said embryogenic culture in a manner such that resulting water potentials of the developing and maturing somatic embryos are reduced below the water potential of the growth environment.

The invention also provides a method of manipulating the availability of water for uptake during the development and maturation of somatic embryos in a culture vessel, which comprises placing an aqueous liquid maturation medium in the vessel, positioning a porous support carrying a culture of the embryos on the liquid medium such that there is no direct contact between the medium and the culture, sealing the vessel with a cover, and allowing said embryos to develop and mature.

The present invention also provides a growth environment suitable for maturing somatic embryos, wherein the water potential of the embryogenic tissue is manipulated to initiate and optimize embryo development and maturation.

Further, the present invention provides somatic embryos prepared according to the manipulation methods and using the growth environments described herein.

By the term "water potential of the embryogenic tissue and/or somatic embryos" the applicants mean the total water potential which is a sum of osmotic potential, turgor potential, and matric potential of the cells of embryogenic tissue and/or somatic embryos.

By the term "matric potential" the applicants mean the effect of water molecules physically binding or adhering to surfaces, on the availability of water for uptake by embryogenic cultures and/or somatic embryos. In connection with a physical support for an embryogenic culture, it will be noted that, the coarser or more porous the material, the less water will be physically bound to the fibers, etc. A more dense or "fine" (i.e. less porous) material will physically bind more water. When comparing equally tall blocks of two materials of different degrees of coarseness, there will be less capillarity in the coarse block than in the fine block. Consequently, water will be drawn up closer to the top of the fine block, and therefore, be more available to a culture supported on the fine block than the coarse block. The result is that the culture on the coarse block will be exposed to more negative water potential and therefore will be under greater water stress than the one on the fine block. The porosity of the material is sometimes referred to as the gradient of the matric water potential.

By the term "water availability", the applicants mean the availability of water for uptake by the maturing embryo, as opposed to water that may be unavailable due to association with a matrix or the like. Water availability can be affected by physical means of control, including (but not limited to) pressure, matric and gravitational effects. The effects of physical means on water availability are separate and distinct from the effects of solutes and their resulting osmotic potentials.

By the term "growth environment", the applicants are referring herein to one or both of the liquid maturation medium, and the physical support (cross-linked polymeric

agents, porous materials, and the like) on which or in which the embryogenic culture is placed. The manipulation and control of the water potential of the embryogenic tissue and/or somatic embryos is achieved without significant changes to the solute concentrations within the maturation medium. In essence, the key to the method of the present invention is the ability to precisely apply, manipulate and control the water potential of the embryogenic tissue and/or somatic embryos during maturation using a physical means. Most commonly, although not necessarily, the water potential of the embryogenic tissue and/or somatic embryos is reduced by the physical means of controlling water availability from the liquid maturation medium. The applicants have chosen the term "physical means" in order to distinguish the manipulation techniques contemplated as being within the scope of the invention from the use of solute manipulation of the maturation medium disclosed in the references discussed above. Accordingly, embryo development using the methods of the present invention, is stimulated without the concomitant disadvantages (i.e., poor embryo quality, poor germination vigour) found when embryo maturation is affected by altering the concentration in the liquid culture medium, of each solute alone or in combination, said solutes including solutes such as PEG, dextran, sugars and the like, whether permeating or non-permeating. In the present invention, certain magnitudes of water potentials within embryogenic tissues and/or somatic embryos can be achieved through physical means that reduce the water potentials below that of the culture medium. This allows precise reductions in the water potentials without increasing the concentration of osmotically active solutes in the liquid medium which is accompanied by negative effects on somatic embryo development, maturation, and germination. It has been found that certain critical magnitudes of water potentials achieved through manipulation of solute concentrations in culture media, interfere with or otherwise impede embryo development and maturation (Klimaszewska et al., 1997).

The physical means of controlling the water potentials of embryogenic tissues and/or somatic embryos may be exerted, for example, by separating the culture from the growth medium by a porous support, or by introducing a gelling agent into the growth medium.

The present invention is applicable during the maturation of somatic embryos from a wide range of plant species, and enables the embryos to be maintained successfully in culture vessels for longer periods of time than has been shown in the methods currently known and practised. This extended maturation stimulates the development of superior, high quality embryos with lower water contents than heretofore has been achieved. In addition, the methods disclosed in the present invention for manipulating the water potentials within embryogenic tissues to affect the initiation and maturation of somatic embryos can be readily practised in conjunction with any somatic embryogenic culture media.

Furthermore, and equally importantly, the somatic embryos prepared using the maturation method described herein are amenable to further drying by desiccation techniques commonly known and practiced in the art, to water content levels that approximate those of natural zygotic seeds. The subsequent germination success rates of somatic embryos produced by the methods described herein also compare favourably to those for natural zygotic seeds.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1A and FIG. 1B are schematic drawings respectively showing a side view and a top plan view of one embodiment.

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of a growth environment as contemplated within this invention, used to obtain the results in Examples 14. The growth environments in these examples consisted of substrates containing fixed volumes of liquid growth medium and varying concentrations of crosslinked-polymeric agents. The embryogenic cultures were separated from the substrate by a thin porous support.

FIG. 2 is a schematic drawing showing one embodiment of a growth environment as contemplated within this invention, used to obtain the results shown in Example 5. The growth environment in this example comprises a porous support with a sloping upper surface that enables positioning embryogenic cultures at different heights above the liquid maturation medium, thereby affecting water availability to the cultures.

FIG. 3 is a schematic drawing showing one embodiment of a growth environment as contemplated within this invention, used to obtain the results shown in Example 6. The growth environment in this example comprises a porous support that enables positioning embryogenic cultures at a specific height above the liquid maturation medium. The availability of water to the cultures can be manipulated by adjusting the height of the porous support placed into the growth environment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods by which a growth environment of a somatic embryogenic culture may be manipulated during the maturation phase in order to control, as precisely as required, the water potential and thereby the water availability to the embryo culture. As already noted, by "growth environment", the applicant is referring herein to one or both of the aqueous liquid maturation medium, and the physical support (cross-linked polymeric agents, porous materials, and the like), if any, on which or in which the embryogenic culture is placed.

It is contemplated within the scope of this invention that water potential of the embryogenic tissue be manipulated during maturation of somatic embryos by one of a number of suitable means as described further herein below. The key to the invention is that this manipulation or control of the tissue water potential is achieved without manipulating the solute concentrations in the medium. Preferably, the solute concentrations are optimized within normal ranges for development of the embryos, and then the water availability to the cultures is manipulated by physical means to stimulate optimum maturation.

Although it is desirable to control (i.e. generally reduce) the amount of water available to a somatic embryogenic culture, the provision of some "free" water to the culture is necessary to enable the essential biological activity required for embryo maturation to occur. "Free" water refers specifically to water molecules that can be directly absorbed by plant cells and incorporated into metabolic pathways and physiological processes. The availability of "free" water, however, does not depend only on the water content of a culture medium; it is a complex function of physico-chemical adsorptive and solution factors. Water adsorbed onto surfaces may or may not be available for absorption by plant cells, depending on how tightly the individual water molecules are adsorbed onto the physical surface of a structure, and on how effective the plant cells are in removing water molecules attached to surfaces. By "surfaces", the applicant is referring to both the surface walls of containers into which culture media is placed, as well as the surfaces of

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any physical supports onto which or within which the embryogenic cultures are placed for the purpose of developing and/or maturing embryos. The points of attachment of water molecules to surfaces are called the meniscus. The effect of adsorption on water activity is often called the "matric effect", the matrix of substances or materials adsorbing the water at the meniscus, is directly responsible for reducing water availability for absorption and incorporation into biological processes by plant cell cultures.

Also, when solutes are dissolved in water, they become more or less hydrated i.e., chemically attached to individual water molecules. Water molecules that become attached to solutes are no longer "free" water molecules but rather, are "bound" or unavailable for incorporation into metabolic pathways. The degree to which solutes become hydrated, e.g., within a culture medium, will effect the availability of "free" water for uptake and incorporation by the embryogenic plant cultures. The effects of solute interactions with individual water molecules, on the availability of "free" water as referred to by the term "water activity," is then called the "osmotic effect."

The ways in which water availability are influenced by adsorption and solution factors may also be referred to as the "water potential." Another way to put it is that water potential is a measure of the specific chemical activity of water which indicates its freedom to interact with or be used by biological systems, and thereby determines water availability.

A somatic embryogenic culture, separated from direct contact with the culture medium, i.e. the source of water and nutrient (solute), by placement on a porous support as contemplated within one embodiment of the present invention, will only have a certain amount of "free" water available to it. The porous material will adsorb water and the avidity of this adsorption is determined by the physical and chemical, i.e., physico-chemical properties of the material. Accordingly, the amount of water available to the embryos supported on a porous medium is a function of one or more of the following:

1. The porosity of the support material i.e., the diameters, lengths and volumes of airspaces, through which liquids and air can flow within the physical structure of the support material.
2. The hydrophilic or hydrophobic properties of the materials comprising the physical support. Porous supports comprised of materials with hydrophilic properties tend to attract and adsorb water molecules resulting in concave-shaped meniscus, while porous supports comprised of materials with hydrophobic properties tend to repel water molecules and form convex meniscus.
3. The height of the support material i.e. the degree of separation of the embryogenic culture from the medium. This is shown by numeral 22 in FIG. 3 and is discussed further below.
4. The volume of liquid medium within the culture vessel (per FIGS. 2 & 3), which is directly related to height as described further below; or the volume of water held in a porous membrane (per FIG. 1) placed between the embryogenic culture and the physical support material. The diameter and length of a pore structure comprised by hydrophilic materials will strongly attract and adsorb water molecules thereby creating a partial vacuum within the pore that will draw water upwards against the forces of gravity; this process is often referred to as cavitation. In contrast, pore structures comprised of hydrophobic materials will repel water and consequently, will not support the formation of partial vacuums within the pore resulting in minimal

water movement upwards as a consequence of cavitation. The diameter and length of the individual pore structures combined with their three-dimensional arrangements and hydrophilic/hydrophobic properties significantly affect the degree of cavitation within the porous support which in turn, directly impacts on the ease and rate, as well as the height of capillary movement of solutions through the porous support.

The porous support may comprise many different types of materials, therefore, it is not intended that the present invention be limited to any one type of hydrophilic or hydrophobic material, or to certain arrays or combinations of physical materials to the exclusion of others. The most important features are that the physical support be "porous" in that it facilitates the movement of solutions through absorption and/or cavitation, that it provides a heterogeneous matrix consisting of solid, liquid and air phases, for support of the embryogenic culture, and that it be non-toxic to the embryogenic culture. Some examples of suitable porous support structures include but are not restricted to:

1. different-sized, regular or irregular-shaped cell-like structures formed through natural or synthetic extrusions as exemplified by, but not restricted to foams or sponges, in which water flows from cell to cell by the processes of absorption and/or adsorption and/or saturation and/or cavitation,
2. regular or irregular interwoven networks of solid tube-like structures, e.g., fibres, of natural or synthetic origin as exemplified by, but not restricted to screens, filters, and absorbant tissues, in which water flows among the networks of solid fibres by the processes of absorption and/or adsorption and/or saturation and/or cavitation,
3. regular or irregular interwoven networks of hollow tube-like structures, e.g., fibres, of natural or synthetic origin as exemplified by, but not restricted to screens, filters, and absorbant tissues, in which water flows within and through the hollow fibres as well as among the networks of hollow fibres by the processes of absorption and/or adsorption and/or saturation and/or cavitation, and
4. regular or irregular interwoven networks of mixtures of hollow and solid tube-like structures, e.g., fibres, of natural or synthetic origin as exemplified by, but not restricted to screens, filters, and absorbant tissues, in which water flows within and through the hollow fibres as well as among the networks of hollow and solid fibres by the processes of absorption and/or adsorption and/or saturation and/or cavitation.

It is preferred that the maturation medium be in liquid form; however, in terms of composition, it may be selected from any basal media known and applied in somatic embryogenesis, including but not limited to modified Litvay medium (Litvay et al. 1985), MSG medium (Becwar et al. 1990) and DCR medium (Gupta and Durzan 1985). Generally, basal media containing sucrose and other osmotic and nutritive solutes are used for the processes inherent in somatic embryogenesis. For maturation of somatic embryos, most commonly, sucrose is used in the range of 0.1 M to 0.4M, and the media consequently typically have osmolalities in the range of approximately 150 to 480 mmol kg⁻¹ (for example, see Klimaszewska et al., 1997). The water potentials of the media in these examples are in the range of -0.37 MPa to -1.20 MPa.

Osmolalities of the media can be adjusted with addition of non-permeating osmotic agents such as PEG in order to reduce water potential to about -1.5 MPa. This commonly results in unpredictable and infrequent somatic embryo maturation, and subsequently, low rates of germination

success (see, for example, the maturation of *Larix* spp. somatic embryos in Klimaszewska et al., 1997; also, see Tables 3, 12 and 13 in this application).

The exact level of water potential which is optimal for each plant species, varies with species. After somatic embryos have been developed and matured in various culture systems including gelled substrates or physical supports containing liquid media, further reductions in water potentials have been employed in the prior art to achieve further desiccation of somatic embryos. The procedures for reducing relative humidities of the air surrounding harvested somatic embryos are well-known (for example the McKersie patent; and 1993 U.S. Pat. No. 5,183,757 to Roberts, the contents of which are incorporated herein by reference, and said patent referred to herein as the Roberts patent). Typically, initial relative humidities of more than 85% are used to create water potentials in excess of -20 MPa. Subsequently, if desired, greater levels of somatic embryo desiccation may be achieved by using relative humidities of approximately 85% or less, which will provide water potentials of -20 MPa or less.

In one embodiment of the present invention, the water potential of the embryogenic tissue is manipulated not by separating the culture from the medium, but by increasing the concentration of a gelling agent in the medium above the level used in the induction and development media. It has been found that as the concentration of gelling agent in the maturation medium increases, the availability of water decreases, thereby imposing reduction in water potential within the embryogenic culture and/or somatic embryos. The magnitude of the water potential within the embryogenic cultures and/or somatic embryos can be precisely controlled by varying the concentration of the gelling agent used to prepare the medium.

A gelling agent, once mixed with a solvent, gives rise to a complex but homogenous physical matrix network in which the water plus the inorganic salts, sucrose, growth regulators, vitamins etc., are trapped. The preferred increase in gelling agent concentration depends, to some extent, on the type of agent used. For gellan gum, this generally means amounts of more than about 0.4%. For agars, this generally means amounts of more than about 0.6% (e.g. MBI-1 agar), 0.8% (Difco-Bacto agar), or 1.0% (MBI-2 agar).

More specifically, it has been found that the concentration of gellan gum (marketed under the names Gelrite® and Phytigel®) may be increased in the maturation medium to within the range of 6 g/l to 12 g/l, most preferably 7 g/l to 10 g/l. In terms of percentages by weight of gellan gum relative to the medium, the preferred concentration is preferably above 0.6%, normally 0.6 to 1.2%, more preferably above 0.8%, and normally about 1.0%. The conventional concentration of gellan gum used in growth media is typically about 0.1 to 0.4%, so it can be seen that the amount of gum used in the present invention is significantly higher.

With respect to agar (marketed under the names Noble®, MBI®, and Difco-Bacto®, among others) the preferred concentration range is between 16 g/l to 20 g/l. It is preferred that the gel strength in the medium fall within the range of 500-1100 g/cm⁻², more preferably from 700-800 g/cm⁻². The applicants have found a significant positive response of the somatic embryos during development and maturation of embryogenic cultures, regardless of the basal medium used, to higher than expected levels of gelling agent.

The amount of gelling agent used in the growth medium depends to some extent on the gelling property of the particular agent. Agents with higher gelling strengths are usually required in lower concentrations. Normally, the

required concentration of gelling agents is such that it results in a gel strength at least 135 g/cm^2 , more commonly $500\text{--}1400 \text{ g/cm}^2$, and most preferably $750\text{--}1400 \text{ g/cm}^2$.

By using gelling agents of increased concentrations as disclosed herein and in conjunction with media of water potential in the range of -0.43 to -0.44 MPa , we provide availability of water that is optimal for increasing mature somatic embryo numbers, quality and desiccation tolerance. The optimal water availability is defined by the water potential of the embryogenic tissue and mature somatic embryos. The range of useful tissue water potential is from -0.20 MPa to -1.20 MPa . (Details of how water potential can be measured are provided in Example 3 below).

The present invention also provides a growth environment suitable for maturing somatic embryos wherein in the water potential of the environment is manipulated by adjusting the water availability within a substrate by adjusting only the concentration of the gelling agent. The nutrients necessary for embryo maturation are added in the form of liquid media at the concentrations known in the art to be appropriate for somatic embryo development, but their concentrations are not manipulated to affect the water potential of the substrate or of the embryogenic culture and/or somatic embryos. With reference to FIGS. 1A and 1B, molten cross-linked gel 1 is dispensed into a container 2 and allowed to solidify, after which a thin porous support 3 comprised of filter paper, filter pads, screens and the like, may be placed onto the cross-linked gel. It is onto the surface of the cross-linked gel or alternatively, onto the thin porous substrate laid on top of the gel, that the embryogenic culture 4 is placed and held during embryo maturation.

Australian Patent Application 37150/93 by Smith (hereinafter, the "Smith Application") discloses a very specific medium composition used for development, maturation and germination of embryogenic cultures, particularly for *Pinus radiata*, in which the solute concentration of the medium is altered. This alteration apparently allows maintenance of the embryogenic cultures without the need to add plant growth regulators such as auxins and cytokinins. Generally, the level of calcium is lower and the levels of total nitrogen, copper, zinc and sodium are increased. Disclosure is made of transiently increasing the gelling agent concentration in the medium in an early phase of maturation.

There are at least two key differences between the Smith Application and the present invention. Firstly, in the Smith Application, the gelling agent concentration is only transiently increased and is not maintained at this higher level for the duration of maturation. The culture is transferred from one medium with a higher concentration of gelling agent to a medium with a lower concentration of gelling agent, all during the maturation phase. With the method of the present invention, exposure to the higher level of gelling agent is continuous. Secondly, the maturation medium disclosed in the Smith Application are very specific in composition and use, as summarized above. In contrast, the method of the present invention contemplates that any media known in the art for maturing somatic embryos, may be manipulated by increasing the gelling agent concentration, to mature somatic embryos.

In another embodiment of the invention, the water potential of the embryogenic tissue and/or somatic embryos is effectively reduced by placing the embryogenic cultures on a porous support within a medium-containing vessel, wherein the support is positioned such that the somatic embryogenic culture is in contact only with medium that is incorporated within the structure of the physical support.

With reference to FIG. 2, which shows example of a suitable growth environment, culture vessel 25 is provided

which may be any conventional petri dish or plate or other suitable container. Disposed within the vessel is a maturation medium 26. A porous support 27 fits within vessel 25 and is in direct contact with medium 26. However, at least one surface 28 of support 27 is separated from direct contact with medium 26. It is on surface 28 that embryogenic cultures are placed for embryo development and maturation. Furthermore, surface 28 of support 27 is provided with an angle such that one end of surface 28 is more elevated about medium 26 than the other end. Thus, the water availability to the embryogenic cultures can be manipulated by the locations on surface 28 where the cultures are placed. With reference to FIG. 2, embryogenic culture placed at location 29 is 2.6 cm from the surface of medium 26, while embryogenic culture placed at location 30 is 3.5 cm from the medium surface, and embryogenic culture placed at location 31 is 4.0 cm from the medium surface 26. Consequently, water availability to the cultures progressively decreases from location 29 to 30 to 31. The culture vessel is then sealed from the environment to provide sterile conditions and optionally, with a further sealing means covering the vessel and lid or cover such as a cling film of plastic material, adhesive tape or the like (not shown).

Another example of a suitable growth environment is illustrated in FIG. 3 which shows an example of a growth environment 10, a culture vessel 12 is provided which may be any conventional petri dish or plate or other suitable container, having a lid or cover 14. Disposed within the vessel is a maturation medium 16. A porous support 18 fits within vessel 12 and is in direct contact with medium 16. However, at least one surface 19 of support 18 is separated from direct contact with medium 16. It is on surface 19 that embryogenic culture 20 is placed during maturation.

In a preferred form and with reference to FIG. 3, liquid maturation medium 16 is placed within the culture vessel 12. Porous support 18 is then placed within culture vessel 12 at least partially in contact with the medium but such that one surface 19 of the support is elevated above and out of direct contact with the medium. The culture vessel is then sealed from the environment by lid 14 to provide sterile conditions and optionally, with a further sealing means covering the vessel and lid or cover such as a cling film of plastic material, adhesive tape or the like (not shown).

The degree of porosity of the material may be chosen according to the particular requirements of each maturation process. This may be readily determined without undue experimentation by a person skilled in this technical field.

Accordingly, any porous natural or synthetic material may be used which provides a gradient of water potential resulting from the height shown in FIG. 3 at 22 (distance between the surface holding the culture and the medium). It is preferred that the support comprise a natural or synthetic open-celled foam or sponge such as, but not restricted to polyurethane foams, e.g. Oasis™ foam, cellulose sponge or pads, pads of rock fibers, e.g. Rockwool™, or pads of fibres such as polyester, nylon, or cellulose. In addition, differential water-permeable membranes or filters may be used.

The appropriate height of the surface of the porous support holding the embryogenic culture is dependent upon the porosity and hence matric potential of the material used to make the support. As an example in the case of Oasis™ foam, it has been found that the preferred height which gives rise to good somatic embryo production by *Picea glauca* is in the range of 10–14 mm above the medium level. This will likely vary for other types of support material and other plant species.

In operation, the somatic embryo culture at the appropriate stage of development (refer to U.S. Pat. Nos. 5,238,835

and 5,563,061 for methods of preparing somatic embryos to the maturation phase, the disclosures of both of which are incorporated herein by reference) is placed on the surface of a porous support. This support may have been previously placed within a culture vessel such as petri dish or plate or other suitable container containing a culture medium, or it may be fitted within the vessel subsequent to the placement of the somatic embryo cultures thereon.

One principal advantage of this maturation method is that water availability in the culture can be precisely controlled without changing the solute composition and concentration, simply by selecting a physical support with certain porosity properties and/or by adjusting the height of the culture medium within the culture vessel. Accordingly, the resulting somatic embryos are of higher quality and show greater germination success rates than embryos produced using other maturation methods. In addition, using this method, the medium can be refreshed without removing the culture from the support. Furthermore, the water potential can be altered very simply by increasing or reducing the level of free liquid medium in the culture vessel, thereby altering the height of the surface of the support holding the culture above the medium. Heretofore, the separation of the somatic embryo culture from the medium by way of a porous support, the matrix of which carries water to and controls its availability to the embryogenic culture has not been contemplated for maturation phase of embryogenesis processes, nor have the attendant advantages been appreciated.

The methods disclosed herein are suitable for use with embryogenic tissue from any plant species without limitation. However, these methods may be used with embryogenic tissue from gymnosperm species, in particular from the gymnosperm plant families Araucaceae, Cupressaceae, Cycadaceae, Ginkgoaceae, Pinaceae, and Taxaceae, and also, from angiosperm species, in particular from the angiosperm plant families Aceraceae, Fagaceae, Hamamelidaceae, Leguminosae, Myrtaceae, Rosaceae, and Salicaceae, and hybrids thereof.

The present invention also provides a growth environment suitable for maturing somatic embryos wherein the water potential of the embryogenic tissue is configured to optimize embryo development and maturation. The growth environment, as discussed in more detail above with respect to the method of operation, comprises, with reference to FIG. 3, culture vessel 12 comprising maturation medium 16 and porous support 18 which is placed with vessel 12 such that surface 19 of the support is separated from direct contact with medium 16. It is on surface 19 that embryogenic culture 20 is held during maturation.

The somatic embryos, matured in accordance with the present invention, may be dried by the methods and techniques disclosed in the McKersie Patent (see background section above) or in PCT Patent Application No. 91/01629 and published on Feb. 21, 1991 (hereinafter, the "BCRI Patent"), the contents of both of which are incorporated herein fully by reference. The McKersie Patent discloses two types of embryo drying techniques: fast drying which is achieved by air drying or in a low relative humidity chamber. Under this regimen, embryos are dried to as low as 7.4% moisture content within a day. Slow drying is achieved by placing the embryos in a series of desiccators with controlled relative humidity for six days. For the first day of drying, embryos are kept at 97% humidity and are transferred daily in succession to chambers with 87%, 75.7%, 62.55%, 50.5% and finally to 43% humidity.

The BCRI Patent discloses partial drying wherein the embryos are exposed to an atmosphere having a relative humidity of between 85–99.9% prior to germination for at least one day.

Accordingly, using the methods disclosed herein and the drying methods incorporated by reference, matured, dried somatic embryos are produced having superior germination frequencies and moisture contents which approximate those of natural zygotic seeds.

EXAMPLE 1

MATURATION OF *Pinus strobus* SOMATIC EMBRYOS ON MEDIA WITH ELEVATED LEVEL OF GELLAN GUM

Three media, MSG (Becwar et al. 1990), ½ LM medium (Litvay et al. 1985) with macroelements reduced to half-strength, and EMM medium (Smith 1994) were used in the experiments. MSG and ½ LM contained 40 mg l⁻¹ iron chelate (7%, Plant Products, Brampton, Ontario, Canada) as iron source. The pH of the media was adjusted to 5.8 prior to sterilization in the autoclave (121° C., 1.25 kg cm⁻², 18 min.). The amino acid solutions were filter-sterilized and mixed into the cooled media. MSG medium was supplemented with 1.46 g l⁻¹ L-glutamine, ½ LM with 0.5 g l⁻¹ L-glutamine and 1 g l⁻¹ casein hydrolysate (CH, Sigma)

Maturation experiments were carried out within a period of 8 months with five embryogenic lines of *Pinus strobus* identified as wp-94-5 and wp-94-7 (both wp-94- lines were maintained in culture for 15 months since initiation) and wp-95-6a, wp-95-7a, and wp-95-9a (the wp-95- lines were maintained in culture for 8 months since initiation).

The somatic embryo maturation experiments were performed by combining embryogenic tissue of one line (from several plates), one week after subculture, in a 50-ml test tube, adding liquid medium without growth regulators and vigorously shaking the tube to break up the clumps of tissue into a fine suspension. Subsequently, 3 ml containing 0.3 or 0.5 g of the suspended embryonal mass were withdrawn with the wide-mouth pipette and placed on the moist filter paper disc (Whatman #2, 5.5 cm in diameter) in Buchner funnel attached to a vacuum pump. A short, low-pressure pulse (5 sec, -4.6 kPa) was applied to remove all the liquid medium and anchor the embryonal mass to the filter paper as a thin layer. Each disc of filter paper with the embryonal mass was subsequently placed on a maturation medium in 10 mm×20 mm Petri dishes and cultured for up to 10 weeks. The cultures were kept under dim light condition at 1.6 μmol m⁻² s⁻¹ from cool white fluorescence lamps (Philips F72T12/CW. 56 Watt) under a 16-h photoperiod at 24±° C.

Three maturation media formulations (½ LM, ½ LMa, MSG and EMM; each containing ABA, 3% sucrose, and gellan gum (Phytigel™, Sigma lot # 83H0854) were prepared in 450-ml aliquots. In ½ LMa, all the components were the same as in ½ LM except for the organic additives; glutamine (0.5 g l⁻¹) and CH (1.0 g l⁻¹) were replaced by amino acid mixture (Smith 1994): glutamine 7.3 g l⁻¹ asparagine 2.1 g l⁻¹, arginine 0.7 g l⁻¹, citrulline 0.079 g l⁻¹, ornithine 0.076 g l⁻¹, lysine 0.0559 g l⁻¹ alanine 0.04 g l⁻¹, and proline 0.035 g l⁻¹. EMM medium was prepared as described in Smith (1994) The pH of the media was adjusted to 5.8 prior to sterilization in the autoclave. The solutions of amino acids and ABA were pH adjusted to 5.8, filter sterilized and added in 50-ml aliquots to the sterile, warm, media. Twenty five ml of the molten media were dispensed to each petri dish (100 mm×15 mm) and left unsealed to solidify in an active laminar air flow unit for 1 day.

Measurements of the gel strengths of the various media were carried out after 1 day after dispensing the media into petri dishes. For each time point, 3 petri dishes were tested.

Gel strength was measured with the TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, N.Y., USA/ Stable Micro Systems, Godalming, Surrey, UK) using a 1.2-cm diameter cylinder probe (acrylic), trigger force 2 g, measured medium depth 2 mm, probe speed 1 mm s⁻¹, and pre/post test speed 3/5 mm s⁻¹. Since there was little variation in readings due to position, the readings were subsequently taken at the center of the petri dish. The gel strength of different media is presented as a ratio correlated to the 1/2 LM IM medium, used for initiation and maintenance of embryogenic cultures, which contains 0.4% gellan gum, 2% sucrose, 9.5 μM 2,4-D, and 4.5 μM BA.

Simultaneously, experiments on the somatic embryo maturation of lines wp-94-7 and wp95-6a were carried out using 1-day-old media. Three petri dishes were used for each maturation treatment. The number of cotyledonary somatic embryos was scored after 7, 8, and 10 weeks, and the embryos were transferred onto germination medium. Cotyledonary somatic embryos were placed horizontally on the surface of 1/2 LM medium containing 0.06 M sucrose and 0.6% gellan gum without growth regulators. The cultures were kept for two weeks under dim light (1.6 μmol m⁻²s⁻¹), 16 h photoperiod, 24±°C. and then transferred to the higher light intensity (47 μmol m⁻²s⁻¹) with the other physical conditions remaining as described. The germinants which displayed elongation of the cotyledons and roots (after 6–8 weeks) were inserted (with the roots down) into fresh medium of the same composition in 7 cmx10 cm magenta boxes (Sigma) containing 40 ml of medium and kept under conditions described above.

Somatic plants that showed epicotyl and root growth were planted in peat: vermiculite (3:1) mix and maintained in a growth chamber under controlled environmental conditions (16 h photoperiod, 20/1° C. day/night temperature, light intensity 170 μmol m⁻² s⁻¹ provided by fluorescent and incandescent lamps). The plants were fertilized twice per week with 100 ppm N applied as 20:20:20 N:P:K (Plant Products, Brampton, Ontario, Canada).

Media were solidified with various concentrations of gellan gum, and the gel strengths were measured and related to the gel strength of 1/2 LM IM medium designated by 1.0 (Table 1). As expected, the relative gel strengths were positively correlated to the concentration of gellan gum in the medium. Media containing relatively high concentrations of the solidifying agent appeared to be "drier" than media which contained lower concentrations of the solidifying agent and consequently, would have less "free" water available for uptake by embryogenic tissues developing somatic embryos. In general, gellan gum at 0.4 and 0.6% formed gels with strength that varied depending on the

medium basal salt composition (Table 1). For example, 0.4% gellan gum in 1/2 LM IM medium resulted in the formation of softer gel than in MSG IM medium, and large differences in gel strength were noted when 0.6% gellan gum was used to solidify 1/2 LM versus EMM medium.

On the other hand, when gellan gum was used at 1% in 1/2 LM, 1/2 LM and MSG media, the difference in gel strength readings were negligible (4.3, 4.2, 4.4, respectively) indicating that at this concentration neither basal salts nor organic nitrogen composition had an effect on the tested gel property.

To evaluate the effect of gellan gum concentration (gel strength) on the development of somatic embryos, the maturation experiment was carried out with two embryogenic lines on the media prepared for the gel strength tests, all containing 3% sucrose and 80 μM ABA (Table 1). The growth of the cultures and the development of the mature embryonal structures strongly depended upon the gel strength and was not influenced by the organic nitrogen composition (see 1/2 LM versus 1/2 LMaa). A clear trend was observed in the tissue proliferation rate of the cultures that ranged from relatively abundant on media with relative gel strength of 0.4–1.3 to moderate on 1.8–2.3 gels and minimal on 3.2–5.1 gels. Upon microscopic examination it was noted that the abundant growth was mainly confined to the suspensor-type cells and callusing of the precotyledonary somatic embryos. Media with high-gel strength supported mainly growth of somatic embryos and maturation. The number of mature somatic embryos was positively correlated to the relative gel strength. However, the two tested lines showed different responses on the corresponding media; line wp95-6a produced mature somatic embryos on most of the tested media whereas line wp-94-7 produced them only on media with gel strength ranging between 3.2 to 5.1. The mature somatic embryos on media with low gel strength (0.7–1.3) were approximately 3 mm in length and those on media with harder gels were slightly shorter (approximately 2.0 mm). Most of the somatic embryos matured after 7 to 10 weeks of culture.

Cotyledonary somatic embryos of line wp95-6a from all the tested 1/2 LM and 1/2 LMaa media were germinated. Of 538 somatic embryos, 347 (64%) displayed elongation of the cotyledons and hypocotyl and growth of the primary root. The germination frequencies for 1/2 LM 0.4, 0.6, 0.8, 1.0, and 1.2% gellan gum were 58, 44, 61, 70, 78%, and for 1/2 LMaa 0.45, 0.6, and 1.0% gellan gum they were 50, 55, and 79% respectively. These results indicated that embryos derived from high gel strength had improved conversion frequencies.

TABLE 1

Relative medium gel strength of different maturation media solidified with gellan gum at various concentrations, calculated relative to the 1/2 LM IM medium with 0.4% gellan gum (1.0) and the maturation response of *P. strobus* somatic embryos. Three hundred mg fresh weight of embryogenic tissue were anchored to the filter paper disc per each of the 3 petri dishes per treatment. Relative gel strengths of the media were measured 1 day after dispensing into the petri dishes.

Medium and supplements	Gellan gum (%)	Relative gel strength*	No. of cotyledonary somatic embryos g ⁻¹ FW embryogenic tissue*	
			wp-95-6a	wp-94-7
1/2 LM IM	0.4	1.0 (0.05)	—	—
MSG IM	0.4	1.5 (0.02)	—	—

TABLE 1-continued

Relative medium gel strength of different maturation media solidified with gellan gum at various concentrations, calculated relative to the 1/2 LM IM medium with 0.4% gellan gum (1.0) and the maturation response of *P. strobus* somatic embryos. Three hundred mg fresh weight of embryogenic tissue were anchored to the filter paper disc per each of the 3 petri dishes per treatment. Relative gel strengths of the media were measured 1 day after dispensing into the petri dishes.

Medium and supplements	Gellan gum (%)	Relative gel strength*	No. of cotyledonary somatic embryos g ⁻¹ FW embryogenic tissue*	
			wp-95-6a	wp-94-7
1/2 LM 3% sucrose + 80 μ M ABA	0.4	1.3 (0.03)	53 (35)	0
1/2 LM 3% sucrose + 80 μ M ABA	0.6	2.3 (0.05)	155 (114)	0
1/2 LM 3% sucrose + 80 μ M ABA	0.8	3.2 (0.32)	180 (32)	30 (3)
1/2 LM 3% sucrose + 80 μ M ABA	1.0	4.3 (0.09)	280 (74)	108 (45)
1/2 LM 3% sucrose + 80 μ M ABA	1.2	5.1 (0.14)	295 (45)	77 (10)
1/2 LM aa 3% sucrose + 80 μ M ABA	0.45	1.1 (0.06)	61 (22)	nt
1/2 LM aa 3% sucrose + 80 μ M ABA	0.6	1.8 (0.07)	78 (11)	nt
1/2 LM aa 3% sucrose + 80 μ M ABA	1.0	4.2 (0.09)	255 (106)	nt
EMM 3% sucrose + 80 μ M ABA	0.45	0.4 (0.04)	0	0
EMM 3% sucrose + 80 μ M ABA	0.6	0.7 (0.04)	15 (15)	0
MSG 3% sucrose + 80 μ M ABA	1.0	4.4 (0.14)	nt	nt

* Numbers are means (\pm SD) of 3 replicates
nt- not tested.

EXAMPLE 2

SOMATIC EMBRYO DEVELOPMENT ON MEDIA WITH VARIED GELLAN GUM CONTENT AND ABA CONCENTRATIONS, AND ON MEDIA CONTAINING PEG:

A further series of experiments was carried out with four embryogenic lines of *Pinus strobus* on 1/2 LM medium with 3% sucrose, various concentrations of gellan gum and ABA (Table 2). The results showed a similar trend as in Example 1 with high mean numbers of cotyledonary somatic embryos observed on media with 1% gellan gum. Interestingly, the results also showed beneficial effect of increased ABA content in the media on the embryo development. At the given concentration of gellan gum, supplementing the medium with progressively higher concentration of ABA resulted in higher numbers of mature somatic embryos with the exception of 1.2% gellan gum where the trend was not as clear. The only line tested on medium with 1% gellan gum but without ABA also produced mature somatic embryos however the number was substantially lower than on the corresponding media with ABA (Tables 1 and 2).

Radicle emergence and growth of the germinants was affected by the maturation medium used to produce the

somatic embryos, with variations in the gellan gum concentrations having more pronounced effects than the changes in ABA concentrations. Somatic embryos that matured on media with 1% gellan gum showed higher incidence of germinants with roots than those derived from media with lower concentration of gellan gum (data not shown).

Another experiment was conducted to compare maturation medium with high concentrations of gellan gum with media containing PEG as a non-permeating osmoticum. Medium supplemented with PEG was gelled with a standard concentration of gellan gum (0.4%) to enable assessment of PEG as an osmoticum versus the physical method of controlling water availability by increasing the gellan gum concentration of the medium. More somatic embryos were produced on medium containing 0.4% gellan gum plus PEG than were produced on medium containing 0.4% gellan gum but no PEG (Table 3). However, media containing elevated gellan gum concentrations produced more somatic embryos than the PEG-amended medium. Furthermore, somatic embryos produced on PEG-amended medium did not produce normal germinants while those produced on media containing 0.8% and 1.0% gellan gum concentrations demonstrated 55% and 95% germination success, respectively (Table 3).

TABLE 2

Maturation and germination of *P. strobus* somatic embryos on 1/2 LM medium with 3% sucrose and different gellan gum and ABA concentrations. The means (\pm SD) were combined for all 4 lines tested; wp-94-5, wp-94-7, wp-95-7a, and wp-95-9a. Three experiments with each line and 3 replicates per each treatment were carried out.

Gellan gum (%), ABA (μ M)	Mean number of mature somatic embryos g ⁻¹ FW embryogenic tissue	No. of somatic embryos germinated/No. of somatic embryos tested
0.45, 80	0	—
0.6, 40	4 (4.0)	Nt
0.6, 60	2 (2.8)	Nt
0.6, 80	7 (8.5)	0/10
0.6, 120	17 (24)	5/20
0.8, 80	30 (28)	25/42

TABLE 2-continued

Maturation and germination of *P. strobus* somatic embryos on ½ LM medium with 3% sucrose and different gellan gum and ABA concentrations. The means (±SD) were combined for all 4 lines tested; wp-94-5, wp-94-7, wp-95-7a, and wp-95-9a. Three experiments with each line and 3 replicates per each treatment were carried out.

Gellan gum (%), ABA (μM)	Mean number of mature somatic embryos g ⁻¹ FW embryogenic tissue	No. of somatic embryos germinated/No. of somatic embryos tested
0.8, 120	59 (45)	21/44
1.0, 0	13 (6)*	Nt
1.0, 40	30 (28)	Nt
1.0, 60	37 (21)	Nt
1.0, 80	68 (64)	35/44
1.0, 120	138 (54)	39/57
1.2, 40	18 (7)	Nt
1.2, 60	22 (4)	Nt
1.2, 80	28 (20)	Nt

* Only line wp-95-6a was tested.

nt - not tested.

Numbers in brackets are S.D.

TABLE 3

Maturation of somatic embryos of eastern white pine (*Pinus strobus*, line 6a) after 9 weeks on ½ LM medium with 3% sucrose, 120 μM ABA, PEG and several concentrations of gellan gum (Phytigel™)

Gellan gum (%), PEG (%)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
0.4 gellan gum, 7.5 PEG	155 ± 65	0*
0.4 gellan gum	0-10	n/a**
0.8 gellan gum	250 ± 90	55
1.0 gellan gum	410 ± 35	95

* No germinants with normal morphology, all the germinants had red, thick hypocotyls, short roots and cotyledons and some of them had split epidermis.

** n/a - not available

EXAMPLE 3

MATURATION OF *Pinus strobus* SOMATIC EMBRYOS ON MEDIUM COMPRISING FOUR SELECTED TYPES OF GELLING AGENTS AND THE WATER POTENTIAL OF EMBRYOGENIC TISSUE AND SOMATIC EMBRYOS MATURED ON MEDIUM SOLIDIFIED WITH GELLAN GUM.

Gellan gum is prepared from a bacterial (*Pseudomonas elodea*) polysaccharide which is composed of glucuronic acid, rhamnose and glucose, and as such, differs from other tissue culture media solidifying agents such as agars. Agars are derived from the sea weeds (agarophytes) and represent a spectrum of closely related polysaccharides belonging to the family of galactans. Furthermore, multi-element analyses of gellan gum (Gelrite, K9A 40, Kelco, USA) and agar (purified, Merck) revealed quantitative and qualitative differences in their inorganic fraction (Sherer et al. 1988). These quantitative and qualitative differences between the two gelling agent types are significant enough to pose a question concerning the possibility of stimulatory effect of certain gellan gum components on the maturation of somatic embryos of *P. strobus*. To determine if this was a viable hypothesis, *P. strobus* somatic embryo maturation was carried out on media gelled with several types of agars and gellan gum. In order to make the comparison meaningful,

gel strengths of all the maturation media were measured and the growth of embryonal masses compared at similar gel strength values on the different types of gelling substrates. Moreover, a simple technique was used to determine the amount of liquid available from the medium to the plated embryogenic tissue at the onset of maturation.

Subsequently, we also investigated if exposure of embryogenic tissues and somatic embryos to different amounts of liquid medium would affect their, i.e., the cultures' and somatic embryos', water potential. First, we measured the water potentials of the different maturation media solidified with various concentrations of gellan gum. Subsequently, we measured the water potentials of the embryogenic tissue and somatic embryos at various time points during the culture periods on the different maturation media.

P. strobus embryogenic culture of line 95-6a was maintained for two years prior to the maturation experiments by biweekly subcultures onto modified Litvay's medium (½ LM, see above) (Litvay et al. 1985) containing 1 g l⁻¹ casein hydrolysate, 0.5 g l⁻¹ L-glutamine, 9.5 μM 2,4-D, 4.5 μM BA, 2% sucrose and 0.4% gellan gum (Phytigel™, Sigma). For the maturation experiments, the tissue was bulked up, collected and plated on the filter papers (Whatman #2, 5.5 cm disc) as previously described (see Example 1). The maturation medium was ½ LM as above except for 3% sucrose and ABA at 80 μM (filter sterilized) as a sole growth regulator, pH 5.8. The cultures were kept at 230±2° C., low light intensity (cool white, fluorescence tubes) 16 h photoperiod for nine weeks prior to scoring the number of mature somatic embryos.

Gellan gum (Phytigel™, Sigma) was tested at 0.4, 0.6, 0.8, 1.0 and 1.2% (w/v). Agars Difco-Bacto® and Difco-Noble® were tested at 0.8, 1.6, 2.0, 2.4 and 2.8% (w/v). Agars MBI #1 and #2 derived from cloned algae and obtained from Marine BioProducts International Corp. (Vancouver, BC, Canada) were tested at 0.6, 1.0, 1.5, 2.0% and 1.0, 1.5, 2.0, 2.5% (w/v) respectively. The latter agars, #1 and #2 differed with respect to the gelling property (gel strength).

The maturation medium was supplemented with various gelling agents, autoclaved at 121° C., 0.12 MPa for 18 min in 250-ml aliquots. After addition of the filter-sterilized solution of L-glutamine and ABA, the medium was dispensed at 25 ml per Petri dish and allowed to cool in the

active, vertical flow laminar hood for 24 h. Three Petri dishes per each of the maturation media were used for measurements of the gel strength 48 h after dispensing. The measurements were taken in the center of the Petri dish using MT-Micro materials tester, (Stable Micro Systems, Surrey, England), probe size 1 cm², trigger force 2 g, measured medium depth 2 mm.

Simultaneously the availability of liquid in the maturation media solidified with gellan gum, agar Difco-Noble® and agar MBI #1 at various concentrations were determined by placing the autoclaved, pre-weighed filter paper discs (Whatman # 2, 5.5 cm) on the surface of the medium. The plates were sealed with Parafilm™ and incubated for 48 h under the same conditions as the embryogenic cultures for maturation of somatic embryos. The filter papers were subsequently weighed and the amount of retained liquid was determined in six Petri dishes per tested medium.

Water potentials of maturation media solidified with various concentrations of gellan gum were measured using a Vapor Pressure Osmometer model 5520 (Wescor, Inc., Utah, USA) according to the protocol outlined in the User's Manual. Briefly, the sample discs (3 per petri dish) were placed on the surface of the gelled medium in the petri dishes. The plates were sealed with Parafilm™ and left for several hours (i.e., from 2 to 24 hrs). No differences were noted in the water potentials of the media after the different equilibration times of the sample discs. The water potential of each sample disc was determined by placing it into the sealed sample chamber (AC-063) for 3 min prior to initiating the measurement cycle. The osmolality unit (mmol/kg) recorded by the machine was subsequently converted to the water potential unit MPa at 25° C.

Twenty to 30 somatic embryos were collected from each maturation medium after 9 weeks and placed on ½ LM medium with 2% sucrose and 0.4% gellan gum in 100×15 mm Petri dishes for germination. The cultures were placed under low light intensity (cool white, fluorescence tubes), 16 h photoperiod. The embryos were scored as germinated if the radicle length was at least 3 mm and the hypocotyl and cotyledons were green and elongated.

Water potentials of embryogenic tissues and somatic embryos were measured with the Vapor Pressure Osmometer model 5520 (Wescor, Inc., Utah, USA) using a larger sample holder (part # AC-064) according to the protocol in the User's Manual for measuring the water potential of large samples. The embryogenic tissue (approximately 20–30 mg fresh mass) or somatic embryos (7 to 12, depending on the size) were collected at various time points during maturation periods on the different maturation media. The samples were placed as quickly as possible in the sample chamber, which was then quickly sealed and left first for 5 min prior to initializing the measurement cycle. After recording the first value, the sample was left in the sample chamber for the next 3 minutes or multiples of the 3 minute period until the consecutive values recorded differed by less than 10 mmol/kg. The osmolality units (mmol/kg) were subsequently converted to the water potential unit MPa at 25° C.

Gel strength in the different media was dependent on the concentration of solidifying agents. Compared to agars, gellan gum formed gels of the highest strength when used at the same concentration (Table 4). For example, to form gel that was similar in strength to 0.8% gellan gum, it was necessary to use approximately 2% of agar Difco-Bacto™ and Difco-Noble™, 1.4% agar MBI #1 and 1.7 % agar MBI # 2.

The numbers of mature somatic embryos were positively correlated to the gel strength of the maturation media and did

not depend on the type of gelling agent used (Table 4). This upward trend in the number of mature somatic embryos was observed on all the maturation media within the range of the gelling agent concentrations tested. Furthermore, the amount of proliferated tissue was visibly diminished on all media with higher gel strength which indicated that these media did not support tissue proliferation but only somatic embryo development. On media with gel strength approximately 800 g cm⁻² and greater (up to 1300 g cm⁻²), most of the embryos reached cotyledonary stage after 9 weeks. However, many younger embryos were still observed. These somatic embryos developed further if left on the medium for a further two to three weeks. All the somatic embryos could be left, if necessary, on the same medium for up to 16 weeks and after becoming cotyledonary, most of them remained developmentally arrested. No greening or germination was noticeable on these media. Contrary to this, media of gel strength below 500 g cm⁻² not only produced fewer mature somatic embryos, but also, some of those embryos became green and showed elongation of the hypocotyl and radicle if left on the medium longer than eight to nine weeks. It is noteworthy that these somatic embryos were developing on the surface of the embryogenic tissue which initially proliferated abundantly, but after five to six weeks, became necrotic.

To test if the solidifying agents used at different concentrations would affect the availability of liquid from the gelled medium to the embryonal masses cultured on the surface of the filter paper, the change in the filter paper weight after incubation on the surface of medium was measured. Three solidifying agents were chosen for this test; gellan gum, agar Difco-Noble™ and agar MBI#1 (Table 5). The mean amount of liquid in the filter papers showed clear negative correlation to the gelling agent concentration. The difference in the amount of liquid present in the filter paper between the lowest (0.4%) and the highest (1.2%) gellan gum concentration was approximately 44 mg. For agar Difco-Noble™, the difference between 0.8 and 2.8% was 73 mg and for agar MBI #1 between 0.6 and 2.0%, it was 48 mg of liquid. Clearly, the embryogenic tissue cultured on the surface of filter papers was exposed to varying amounts of liquid at the onset of the culture. It is worthwhile to note that the liquid content in the filter papers was similar when compared among media of similar gel strength but gelled with different gelling agents (Table 5). Gels of similar strength values were formed by 0.8% gellan gum, 2.0% agar Difco-Noble and 1.4% agar MBI #1 (estimated) resulting in approximately 350 mg of liquid per filter paper.

There were similarities in the number of mature somatic embryos and germination frequency among the tested gelling agents if applied to give a similar medium gel strength and water availability. Therefore, it is concluded that the amount of water available to the embryogenic cultures placed on the maturation medium was a critical factor involved in the development of somatic embryos of *P. strobus*.

The water availability from the maturation medium had a significant effect on the water potential of the embryogenic tissue which in turn triggered and/or maintained the matu-

ration process (Table 6). After one week of culture, the embryogenic tissue water potential was the same on all the tested media regardless of the gellan gum concentration and it was in an equilibrium with the water potential of the medium. All media solidified with 0.4 to 1.0% gellan gum had water potential of -0.43 ± 0.01 MPa. Two weeks after the embryogenic cultures were placed on the media, trends in the changes of the water potentials within the embryogenic tissues cultured on various media became obvious. The embryogenic tissue cultured on media with 0.4 and 0.6% gellan gum had higher (less reduced) water potential than embryogenic tissue cultured on media with 0.8 and 1.0% gellan gum. This trend in the tissue water potential was maintained through week 4 of the culture. At week 6/7 mature somatic embryos developed on medium with 0.4 and 0.6% gellan gum and their water potential either remained the same as of the embryogenic tissue at week 4 (for 0.6% gellan gum) or increased, particularly on medium with 0.4% gellan gum. At this time, the somatic embryos developing on medium with 0.8 and 1.0% gellan gum were not yet mature (most were precotyledonary). At week 8/9, the somatic embryos on media with 0.4 and 0.6% gellan gum began to germinate or became hyperhydric while on media with 0.8 and 1.0% gellan gum, the somatic embryos reached cotyledonary stage. The water potentials of these mature somatic embryos were much lower than those which matured on media with lower concentrations of gellan gum. These low water potentials within somatic embryos on medium with 0.8 and 1.0% gellan gum were maintained through week 10 and 12.

TABLE 4

Maturation and germination response of <i>Pinus strobus</i> somatic embryos on 1/2-LM medium with 3% sucrose, 80 μ M ABA and solidified with several concentrations of various gelling agents. Two hundred mg FW tissue were anchored to the filter paper disc for each of the 3 petri dishes per treatment. Numbers are means \pm SD.			
Gelling agent (%)	Gel strength (g cm ⁻²)	Number of somatic embryos g ⁻¹ FW tissue	Germination (%)
Gellan gum PHYTAGEL™			
0.4	317 \pm 18	65 \pm 20	nt
0.6	501 \pm 7	nt	nt
0.8	767 \pm 44	160 \pm 15	88
1.0	1072 \pm 75	315 \pm 60	92
Agar Difco-Bacto™			
0.8	135 \pm 2	55 \pm 35	nt
1.6	552 \pm 22	220 \pm 115	39
2.0	806 \pm 34	320 \pm 70	85
2.4	982 \pm 3	nt	nt
2.8	1277 \pm 17	nt	nt
Agar Difco-Noble™			
0.8	143 \pm 1	2.5 \pm 3.5	nt
1.6	569 \pm 8	170 \pm 40	100
2.0	750 \pm 81	290 \pm 40	85
2.4	986 \pm 3	nt	nt
2.8	1345 \pm 7	nt	nt
Agar MBI-1			
0.6	183 \pm 5	0.5 \pm 0.7	nt
1.0	437 \pm 16	16 \pm 3	90
1.5	842 \pm 36	28 \pm 3	86
2.0	1330 \pm 44	45 \pm 14	89

TABLE 4-continued

Maturation and germination response of <i>Pinus strobus</i> somatic embryos on 1/2-LM medium with 3% sucrose, 80 μ M ABA and solidified with several concentrations of various gelling agents. Two hundred mg FW tissue were anchored to the filter paper disc for each of the 3 petri dishes per treatment. Numbers are means \pm SD.			
Gelling agent (%)	Gel strength (g cm ⁻²)	Number of somatic embryos g ⁻¹ FW tissue	Germination (%)
Agar MBI-2			
1.0	265 \pm 10	30 \pm 20	nt
1.5	565 \pm 28	105 \pm 15	90
2.0	937 \pm 15	250 \pm 15	91
2.5	1323 \pm 21	210 \pm 25	95
nt - not tested			

TABLE 5

Liquid content in the filter paper discs placed (for 48 h) on the surface of 1/2 LM medium with 3% sucrose, 80 μ M ABA and solidified with several concentrations of selected gelling agents and the maturation response and germination frequency of <i>Pinus strobus</i> somatic embryos. Two hundred mg FW of tissue was anchored to the filter paper disc for each of the 3 petri dishes per treatment.			
Gelling agent (%)	Liquid content in 227 mg filter paper (mg)	No of somatic embryos g ⁻¹ FW tissue	Germination (%)
Gellan gum (Phytigel™)			
0.4	367.8 \pm 6.9	30 \pm 14	nt
0.6	355.0 \pm 5.8	85 \pm 42	30
0.8	352.8 \pm 7.5	112 \pm 32	70
1.0	336.2 \pm 3.9	300 \pm 65	65
1.2	323.4 \pm 5.3	475 \pm 205	nt
Agar Difco-Noble™			
0.8	385.4 \pm 5.1	17 \pm 3.5	nt
1.6	362.4 \pm 4.9	252 \pm 45	40
2.0	344.5 \pm 7.3	370 \pm 10	65
2.4	322.7 \pm 5.4	260 \pm 42	70
2.8	312.3 \pm 7.6	262 \pm 45	75
Agar MBI #1			
0.6	378.0 \pm 13.0	0	nt
1.0	371.6 \pm 8.8	22 \pm 25	nt
1.5	339.0 \pm 3.2	142 \pm 60	44
2.0	330.4 \pm 15.8	160 \pm 7	53

Numbers are means \pm SD
nt - not tested

TABLE 6

Water availability of *Pinus strobus* (line 6a) embryogenic cultures and somatic embryos on ½ LM maturation medium with 3% sucrose, 120 µM ABA and various concentrations of gellan gum (Phytigel™).

Water potential (MPa)

Gellan gum (%)	1 wk	2 wks	4 wks	6/7 wks	8/9 wks	10 wks	12 wks
0.4	-0.44 ± 0.01	-0.37 ± 0.03	-0.39 ± 0.04	-0.26 ± 0.04	precocious	n/a	n/a
0.6	nt	-0.33 ± 0.04	-0.32 ± 0.05	-0.32 ± 0.02	precocious	n/a	n/a
0.8	nt	-0.43 ± 0.01	-0.45 ± 0.04	not matured	-0.5 ± 0.02	-0.54 ± 0.08	-0.55 ± 0.06
1.0	-0.45 ± 0.01	-0.44 ± 0.03	-0.47 ± 0.02	not matured	-0.66 ± 0.06	-0.67 ± 0.06	-0.70 ± 0.00

Note: From 1 to 4 wks the water potential measurements were made on embryogenic tissue plus developing somatic embryos. From week 6, the water potentials were determined in somatic embryos only.

nt - not tested

n/a - not available.

EXAMPLE 4

MATURATION OF CONIFER SOMATIC EMBRYOS (other than *Pinus strobus*) ON MEDIUM (½ LM) COMPRISING DIFFERENT CONCENTRATIONS OF GELLING AGENT, PEG AND ABSCISIC ACID (ABA) AND WATER POTENTIAL OF THE EMBRYOGENIC TISSUE AND SOMATIC EMBRYOS.

From the previous three examples it is evident that using maturation medium with increased gel strength, than is usually used in the maintenance phase of somatic embryogenesis, has a beneficial effect on the maturation of *P. strobus* somatic embryos with respect to the number of somatic embryos per FW embryogenic tissue, germination and plant conversion frequency. This beneficial effect is due to the concomitant decrease in water availability to the embryogenic tissues which is manifested in reduced water potential in the embryogenic tissue and developing somatic embryos.

To determine if maturation medium with increased gel strength would procure similar response in other than *Pinus strobus* conifer species, a series of maturation experiments was carried out with a number of conifer embryogenic tissues on ½ LM medium gelled with agar or gellan gum at various concentrations and supplemented with several levels of ABA. In some of the species a comparison was made between medium with high gel strength versus semi-solid (0.4% gellan gum) or liquid medium containing PEG MW 4000 as additional solute. Handling of the embryogenic tissues for the maturation experiments and the culture technique were the same as described in Example 1. All the cultures were maintained on ½ LM medium with 0.4% gellan gum (Phytigel™, Sigma) by subculturing onto fresh medium every 14 days. For the maturation experiments cultures not older than 10 days, preferably seven days old were used. The tissue was not subcultured during the duration of maturation. Mature somatic embryos were germinated on ½ LM medium with 2% sucrose and 0.4% gellan gum (Phytigel™). Germination vigor was evaluated after 3 weeks. Water potential measurements were done on embryogenic tissue and somatic embryos of Douglas fir and interior spruce cultured on maturation media with various concentrations of gellan gum (Phytigel™).

Pseudotsuga menziesii:

Table 7 shows results on somatic embryo maturation and germination of line 5001. The number of mature somatic

embryos was positively correlated to the increased concentration of agar or gellan gum in the medium. Somatic embryos matured on media with high gelling agent concentration attained high germination frequencies. No maturation of somatic embryos occurred on medium with 0.4% gellan gum.

Table 8 shows water potential values of embryogenic tissue and somatic embryos when cultured on medium with varied concentrations of gellan gum. Similarly to *Pinus strobus* (as described in Example 3), the water potential of embryogenic tissue after one week of culture was in an equilibrium with the water potential of the media, -0.44±0.04 MPa and -0.43±0.01 MPa respectively. After 2 weeks, clear trends in the water potential of embryogenic tissues were established. The tissue cultured on maturation medium with 1.0% gellan gum had significantly lower water potentials than those of tissues cultured on lower gellan gum concentrations. This trend was maintained through week 4 and by week 8, the water potential of mature somatic embryos from medium with 1.0% gellan gum was significantly lower than those from medium with lower gellan gum concentrations. At week 10, the trends remained the same.

Pinus banksiana:

Table 9 shows the effects of manipulating the water potential of the growth environments on somatic embryo maturation and germination of *Pinus banksiana* line 545. Five concentrations of gellan gum (Phytigel), and three concentrations of ABA were tested with each gellan gum concentration. A clear upward trend in the numbers of mature somatic embryos produced on media containing increased concentrations of gellan gum was observed. It was also beneficial to increase ABA level from 40 µM to 80 µM. Relatively high frequency of germination (>70%) was achieved from somatic embryos that matured on medium with 1.0 or 1.2% gellan gum.

Pinus taeda:

Table 10 shows the effects of manipulating the water potential of the growth environments on somatic embryo maturation and germination of *Pinus taeda* line A. Three gellan gum concentrations each with 120 µM ABA were tested in the maturation media. Medium with gellan gum at 0.8% supported maturation of relatively high number of somatic embryos that displayed germination frequency of over 50%. As gellan gum was further increased, the somatic embryos displayed lower germination frequency.

Picea glauca x *engelmannii*:

Tables 11 and 12 show the effects of manipulating the water potential of the growth environments on somatic embryo maturation and germination of *Picea glauca* x *engelmannii* lines 4-2809 and 10-1418. Liquid medium with PEG 4000 and 60 μ M ABA was tested against semi-solid media gelled with different concentrations of gellan gum (Phytigel™) and 60 μ M ABA. The numbers of mature somatic embryos were always higher on media solidified with gellan gum compared to liquid medium with PEG. In order to test PEG at 15%, it was necessary to use liquid medium because upon addition of gellan gum, the medium would not solidify. The most pronounced effect of both media (liquid with PEG versus gelled medium without PEG) was manifested in the germination response. Low numbers or no normal germinants were recovered from somatic embryos matured on PEG medium (Table 12) and those that

TABLE 7

Maturation of somatic embryos of Douglas fir (<i>Pseudotsuga menziesii</i> , line 5001) after 10 weeks on 1/2 LM medium containing 3% sucrose, 120 μ M ABA and various concentrations of gelling agents.		
Gelling agent (%)	No. of somatic embryos g ⁻¹ FW tissue	Germination (%)
Agar Difco-Noble ®		
0.8	0	n/a
1.6	25	95
2.0	200	97
Gellan gum Phytigel™		
0.4	0	n/a
0.8	>250	>90
1.0	>250	>90

TABLE 8

Water potential of Douglas fir (<i>Pseudotsuga menziesii</i> , line 5001) embryogenic cultures on 1/2 LM maturation medium containing 3% sucrose, 120 μ M ABA and various concentrations of gellan gum (Phytigel™)					
Water potential (MPa)					
Gellan gum (%)	1 wk	2 wks	4 wks	8 wks	10 wks
0.4	-0.46	-0.25 \pm 0.06	-0.16 \pm 0.00	no maturation	no maturation
0.6	-0.38	-0.29 \pm 0.04	-0.27 \pm 0.04	-0.22 \pm 0.04	precocious
0.8	-0.46	-0.39 \pm 0.06	-0.34 \pm 0.03	-0.36 \pm 0.07	-0.38 \pm 0.02
1.0	-0.45	-0.56 \pm 0.05	-0.52 \pm 0.02	-0.50 \pm 0.04	-0.55 \pm 0.05
Mean	-0.44 \pm 0.04				

Note: From 1 to 4 wks the water potential measurements were made on embryogenic tissue plus developing somatic embryos. From week 6 the water potential was determined in somatic embryos only.

matured on medium with 0.25% gellan gum as opposed to the media with high gellan gum level (0.75%) (Table 11).

Table 13 shows water potential of embryogenic tissue and somatic embryos when cultured on media with varied concentrations of gellan gum. Similarly to *Pinus strobus* and *Pseudotsuga menziesii*, interior spruce embryogenic tissue cultured on the maturation media solidified with various concentrations of gellan gum showed decreased water potential on media with high concentration of gellan gum (0.6 and 0.7% versus 0.4%). In this species however, the trend was established sooner than in the other two species because it was distinct after the first week of culture as opposed to 2 weeks. While the embryogenic tissue and mature somatic embryos displayed high water potential on medium with 0.4% gellan gum, the water potentials in cultures grown on medium with high gellan gum were significantly lower.

Picea sitchensis:

Table 14 shows results on somatic embryo maturation and germination of line FB2-253 on medium gelled with gellan gum with and without PEG. High numbers of mature somatic embryos were obtained on all the media however the highest germination frequency was attained from somatic embryos matured on medium without PEG. Somatic embryos matured on either 0.6 or 0.7% gellan gum germinated at 80-90%.

TABLE 9

Maturation of somatic embryos of jack pine (<i>Pinus banksiana</i> , line 545) after 8 weeks on 1/2 LM medium with 3% sucrose, different concentrations of ABA and gellan gum (Phytigel™).			
Gellan gum (%)	ABA (μ M)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
0.4	0	0	n/a
0.4	80	0	n/a
0.4	120	0	n/a
0.6	40	0	n/a
0.6	60	0	n/a
0.6	80	0	n/a
0.7	40	10	n/a
0.7	60	33	>50
0.7	80	66	>50
1.0	40	>165	>70
1.0	60	>165	>70
1.0	80	>165	>70
1.2	40	>165	>70
1.2	60	>165	>70
1.2	80	>165	>70

TABLE 10

Maturation of somatic embryos of loblolly pine (<i>Pinus taeda</i> , line A) after 10 weeks on 1/2 LM medium with 3% sucrose, 120 μ M ABA and various concentrations of gellan gum (Phytigel™).		
Gellan gum (%)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
0.4	43	31
0.8	185	57
1.0	162	33

TABLE 11

Maturation of somatic embryos of interior spruce (<i>Picea glauca</i> \times <i>engelmannii</i> , line 4-2809) after 6 weeks on 1/2 LM medium with 3% sucrose, 60 μ M ABA and gellan gum (Phytigel™).		
Gellan gum (%)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
0.25	752 \pm 330	27 \pm 14
0.5	600 \pm 185	65 \pm 6
0.75	514 \pm 99	70 \pm 16

TABLE 12

Maturation of somatic embryos of interior spruce (<i>Picea</i> \times <i>engelmannii</i> , line 10-1418) after 9 weeks on 1/2 LM medium with 3% sucrose, 60 μ M ABA and gellan gum (Phytigel™) and on liquid 1/2 LM medium with 60 μ M ABA, 3% sucrose and PEG 4000. On the latter medium the tissue was placed on the filter paper which was placed on the nylon screen (500 μ m pore size) which was placed over container with liquid medium in such a way that the nylon screen was touching the surface of the medium.		
Gellan gum (%), PEG (%)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
Gellan gum		
0.4	500 \pm 130	n/t
0.6	805 \pm 55	n/t
0.7	950 \pm 125	91 \pm 8
Liquid		
7.5 PEG	40	7
15 PEG	145	0

TABLE 13

Water potential of interior spruce (<i>Picea glauca</i> \times <i>engelmannii</i> , line 10-1418) on 1/2 LM maturation medium with 3% sucrose, 60 μ M ABA and various concentrations of gellan gum (Phytigel™).					
Water potential (MPa) Gellan gum (%)	1 wk	2 wks	4 wks	6 wks	8 wks
0.4	-0.47 \pm 0.05	-0.33 \pm 0.06	-0.31 \pm 0.01	-0.27 \pm 0.02	-0.20 \pm 0.04
0.6	-0.52 \pm 0.02	-0.47 \pm 0.03	-0.47 \pm 0.02	se not mature	-0.54 \pm 0.06
0.7	-0.50 \pm 0.02	-0.51 \pm 0.02	-0.50 \pm 0.02	se not mature	-0.57 \pm 0.07

Note:

From 1 to 4 wks the water potential measurements were made on embryogenic tissue plus developing somatic embryos. From week 6 the water potential was determined in somatic embryos only.

TABLE 14

Maturation of somatic embryos of sitka spruce (<i>Picea sitchensis</i> , line FB2-253) after 9 weeks on 1/2 LM medium with 3% sucrose, 60 μ M ABA and gellan gum (Phytigel™).		
Gellan gum, (%), PEG (%)	No. of mature somatic embryos g ⁻¹ FW tissue	Germination (%)
0.6 gellan gum	310 \pm 95	83 \pm 7
0.7 gellan gum	240 \pm 110	92 \pm 10
0.4 gellan gum, 5 PEG	315 \pm 270	33 \pm 6
0.4 gellan gum, 8 PEG	430 \pm 60	40 \pm 5

EXAMPLE 5

A test was carried out using apparatus as shown in FIG. 2 of the drawings to determine the effect of height from the liquid level in the type of apparatus that uses a porous support to manipulate the water potential.

The apparatus consisted of an enclosed container 25 holding a body 26 of liquid medium having a depth of about 2 cm. Positioned within the container was a block of dense porous foam material 27 having a sloping upper surface 28 positioned above the upper level of the body of growth medium. Embryogenic tissue was placed on the sloping upper surface at three positions 29, 30 and 31 (referred to below as Position 1, Position 2 and Position 3, respectively). The positions were chosen so that the samples were located, respectively, 2.6 cm, 3.5 cm and 4.0 cm above the upper level of the liquid medium. After a period of culturing, the number of embryos produced from each sample of embryogenic tissue were counted. The results are as shown in Table 15.

The results show that the number of embryos increased as the height above the liquid increased for the first two positions, but decreased for the third position. This may be because there is a critical height at which the capillary action of the foam pores can no longer draw up sufficient nutrient for embryo maturation. This indicates that there is an optimum spacing above the liquid that reduces the water potential sufficiently, while still allowing sufficient nutrient absorption for proper embryo maturation. The optimum height may be determined from experiments such as the above. Clearly, embryo maturation apparatus would be

designed to provide the support surface at the optimum height, which is likely to depend on the porosity and cavitation properties of the physical support material and perhaps the plant species of the embryos concerned and the solute composition of the liquid medium.

TABLE 15

Somatic embryos produced on the sloping surface of a porous solid substrate; cultures were positioned at three different heights above the liquid growth medium.

Slant Format (dense foam)	Position 1	Position 2	Position 3
Height Above Media (cm)	2.6	3.5	4.0
Embryos Produced (#)	25	90	4

NB: Position 1 is closest to the medium while Position 3 is farthest from the medium

EXAMPLE 6

A test similar to that reported in Example 5 was carried out, except for using three blocks of coarse foam material (referred to below as Block 1, Block 2 and Block 3), each having horizontal upper surfaces and different thicknesses, resulting in different heights of the upper surfaces from the liquid level, as shown in FIG. 3. Samples of embryogenic tissue were placed on each foam block and the number of developed embryos were counted after a suitable period of maturation. The results are shown on Table 16.

Again, a similar effect of increased embryo production with height up to an optimum height was observed. However, in this case, the optional height is lower than the optional height on the dense foam support used in Example 5, due to the increased porosity and subsequent decreased capillarity in the coarse foam.

TABLE 16

Somatic embryos produced on the horizontal surfaces of porous solid substrates.

Horizontal Format (coarse foam)	Block 1	Block 2	Block 3
Height Above Media (cm)	1	2	3
Embryos Produced (#)	59	78	6

We claim:

1. A method of developing and maturing plant somatic embryos in a growth environment having a water potential relative to the embryos, which method comprises exposing an embryogenic culture of embryogenic tissue or developing and maturing embryos to a maturation medium, and allowing said embryogenic culture to develop into mature somatic embryos characterized in that a physical means is associated with the medium to reduce the availability of water in the growth environment for uptake by said embryogenic culture, wherein the physical means is selected from the group comprising (i) a gelling agent in a strength of at least about 800 g cm⁻², and (ii) a porous support separating said culture from direct contact with said medium and providing to embryos in contact therewith a water stress.

2. A method according to claim 1, characterized in that the embryogenic culture is from a genus of angiosperm species.

3. A method according to claim 1, characterized in that the embryogenic culture is from a genus of gymnosperm species.

4. A method according to claim 1, characterized in that the embryogenic culture is selected from the genus *Pinus*, and the availability of water is reduced such that the resulting water potentials of the embryogenic tissue or developing and maturing embryos are less than -0.20 MPa.

5. A method according to claim 4, characterized in that said resulting water potentials are in the range of -0.43 MPa to -0.70 MPa.

6. A method according to claim 1, characterized in that the embryogenic culture is selected from the genus *Picea*, and the availability of water in the growth environment is reduced such that the resulting water potentials of the embryogenic tissue or developing and maturing embryos are less than -0.20 MPa.

7. A method according to claim 6, characterized in that the resulting water potentials are in the range of -0.43 MPa to -0.70 MPa.

8. A method according to claim 1, characterized in that the embryogenic culture is selected from the genus *Pseudotsuga* and the availability of water in the growth environment is reduced such that the resulting water potentials of the embryogenic tissue or developing and maturing embryos are less than -0.20 MPa.

9. A method according to claim 8, characterized in that the resulting water potentials are in the range of -0.43 MPa to -0.70 MPa.

10. The method of claim 1, wherein the physical means of affecting the availability of water in the growth environment comprises placing the embryogenic culture on a porous support within a culture vessel, said support being positioned in a liquid medium within the vessel such that the culture is not in direct contact with the liquid medium.

11. A method according to claim 1, further comprising increasing the concentration of gelling agent in the maturation medium without affecting the concentrations of solutes within the medium.

12. A method according to claim 1 wherein the physical means is a porous support within a culture vessel and further comprising manipulating the availability of water for uptake during the development and maturation of somatic embryos in a culture vessel, which comprises placing a liquid maturation medium in the vessel, positioning a porous support carrying a culture of the embryos on the liquid medium such that there is no direct contact between the medium and the culture, sealing the vessel with a cover, and allowing said embryos to develop and mature.

13. A method of claim 1 characterized in that the porous support is selected from the group consisting of natural and synthetic foams, sponges, fibres, membranes and filters.

14. A method of claim 1 characterized in that said porous support is made of a material having a gradient of matric water potential that results in a suitably reduced water potential for proper development and maturation of the embryos.

15. A method of claim 1 characterized in that the availability of water for uptake within the culture vessel is manipulated by the separation of the embryo culture from a liquid medium, the availability of said water being controllable by the porosity of a material of which the porous support is formed, and the height of the culture above the liquid medium.

16. The method of claim 12, wherein the somatic embryos are from genera of angiosperm species.

17. The method of claim 12, wherein the somatic embryos are from genera of gymnosperm species.

18. A method according to claim 1, additionally comprising the steps of removing the embryos from the maturation medium and drying the embryos in an atmosphere having a relative humidity less than 99.9%.

19. A method according to claim 1, additionally comprising the steps of removing the embryos from the maturation medium and drying the embryos in an atmosphere having a relative humidity from 85% to 99%.

20. A method according to claim 1, characterized in that the somatic embryos are selected from the species *Pinus radiata*.

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21. A method according to claim 1, characterized in that the gelling agent is selected from the group consisting of gellan gum, agar, agarose, and cross-linked alginates.

22. A method according to claim 11, characterized in that the gelling agent is gellan gum of a concentration between about 0.9% to about 1.2%.

23. A method according to claim 1, wherein the physical means is a porous support within a culture vessel, said support being positioned in a liquid medium within the vessel such that the culture is not in direct contact with the liquid medium.

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24. A method of developing and maturing somatic embryos, the method comprising maturing an embryogenic culture of embryogenic tissue or developing and maturing embryos in the presence of a suitable maturation medium, characterized in that the medium comprises a gelling agent in a strength of at least about 800 g cm⁻² for reducing the water available for uptake by the embryos.

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